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**בקשה לפטנט**

Application For Patent

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אני, (שם המבקש, מענו ולגבי גוף מאגדת מקום התאגדותו)  
I, (Name and address of applicant, and in case of body corporate-place of incorporation)

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Technion Research and Development Foundation Ltd., Israeli Company of Senate House, Technion City, Park Gootwirt, Haifa 32000, Israel

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(בעברית)  
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Detection of an entity in a sample

(באנגלית)  
(English)

Hereby apply for a patent to be granted to me in respect thereof.

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Detection of an entity in a sample

Technion Research and Development Foundation Ltd.

מוסד הטכניון למחקר ופיתוח בע"מ

C. 110618

## DETECTION OF AN ENTITY IN A SAMPLE

### FIELD OF THE INVENTION

The present invention concerns a device, a system, a kit and a method for assaying a target (for example a biological entity) in a sample.

### 5 BACKGROUND OF THE INVENTION

Detection of biological moieties, such as biological molecules, bacteria, viruses and cells, in a sample is a routine procedure in fields such as medicine, industry and defense. In medicine, detection is routinely carried out for monitoring clinically and bio-chemically important analytes in a sample  
10 obtained from a patient which may be a blood sample, urine sample, etc.

Typically, detection of biological molecules, such as proteins, is carried out by employing well known immunoassay techniques, such as ELISA, radio-immuno assays, etc.

Assays for the presence of specific DNA or RNA sequences in  
15 a sample have various applications including the detection of microorganismal infections in patients, analysis of food or environmental samples to detect a contamination, detection of genetic diseases caused by mutations, etc. Simultaneous detection of a large number of different nucleic acid sequences became important in genome projects, i.e. sequencing of the  
20 full genomes of various organisms, particularly in the human genome project. Such sequencing typically involves the detection of a large number of short, partially overlapping nucleic acid sequences and based thereon constructing a full genetic map. Such a technique which is termed "*sequencing by hybridization*" (SBH), involves the digestion of long DNA molecules into  
25 smaller fragments and their subsequent hybridization with an array of short probes.

The presence of a specific DNA or RNA sequence in a sample can be detected by a labeled probe capable of specific hybridization with the DNA or RNA sequence. However, direct detection by a probe is limited in present techniques to relatively high concentrations of the target DNA or RNA. In order to overcome this problem, methods for amplifying nucleic acid sequences have been developed, including PCR (polymerase-chain-reaction), LCR (ligase-chain-reaction) and 3SR (self-sustained sequence replication). All amplification methods consume substantial time and labor, require specific conditions for the activity of the amplifying enzymes and intricate laboratory apparatus.

Attempts have been made to develop electrochemical sensors which can directly measure the concentration of an analyte in a sample. The sensors generally detect a change in physical, electrical or optical properties as a result of interaction with the analyte.

U.S. 4,314,821 discloses a system for detection of antibodies in a sample based on a change in resonance frequency of piezoelectric oscillators, as antibodies bind to the oscillator. U.S. 4,822,566 discloses an apparatus for detecting the presence and/or measuring the concentration of an analyte in a fluid medium, relying on the bio-specific binding of the analyte to a biochemical host system thus modifying the dielectric properties of the sensor.

U.S. 5,312,527 discloses a voltammetric sensor for the detection of a target polynucleotide sequence in a sample which binds to a complementary sequence immobilized on an amperometric electrode. A change in the electrode's electric response then indicates the presence of the target sequence in the sample and includes means for detecting, voltammetrically, immobilized heteroduplexes.

WO 9744651 discloses an apparatus for the detection of a specific nucleic acid sequence in a sample, which involves the use of a

biosensor comprising an electrode and a bilayer lipid membrane. The membrane is composed of modified lipid molecules which are assembled into an electrode/ionic reservoir/insulating bilayer combination that is suitable for incorporation of ion channels and ionophores. The conductance of such a  
5 membrane is dependent on the presence, or absence of an analyte. The presence of the specific nucleic acid sequence changes the impedance of the membrane which is then measured.

## GLOSSARY

10 In the following, use will be made with some terms, some of which and their meaning are as follows:

*Biological entity* – an entity which is derived from a biological source and may be a biological molecule (nucleic acid, protein, lipid, antibody, hormone,  
15 etc.) a complex of several biological molecules, a bacterium, a virus, a cell (of a multicellular or unicellular organism) a cell organelle (nucleus, nucleosome, mitochondria).

*Affinity group* – a group of at least two entities (biological or non-biological),  
20 capable of *binding* (see below) to each other. Examples of affinity groups are: two complementary strands of nucleic acid sequences; antibody-antigen, ligand-receptor; enzyme-substrate, glycoprotein-lectin, bacterium and its antibody; DNA - DNA binding protein; etc. An example of an affinity group containing a non-biological entity is an antibody and its specific  
25 non-biological hapten.

*Binding* - non-covalent specific interactions (ionic, van der Waals, hydrogenic, hydrophobic, etc.) between at least two members of an affinity group, for example, the interactions between a nucleic acid sequence and its

specific complementary sequence, the interaction between an antibody and its antigen, etc.

*Target* – an entity, the presence of which is to be assayed in a sample. The  
5 target, which may be biological or non biological is a member of an affinity group. The target may be an entity *per se* which is to be detected or at times such an entity bound to or complexed with a modified agent that facilitates the binding of said entity to a *recognition moiety* (see below).

10 *Recognition moiety* - a member of an affinity group, the group including the target and at least one other entity capable of binding thereto, and which is used for the detection of target in the sample. The recognition moiety may be *attached* (see below) to an *electrode* (see below) or to another substrate.

15 *Binding moiety* – a term referring collectively to either a recognition moiety or a moiety which can bind to the target in a non-specific manner or in a semi-specific manner (semi-specific binding meaning binding to a group of entities which display a common characteristic, e.g. binding to molecules with a specific charge, binding to mRNA in general, binding to a class of  
20 antibodies, e.g. to all human IgG antibodies, etc.).

*Attachment (attach)* – the interaction between the recognition or binding moiety and a substrate (for example the electrode) whereby said moiety becomes immobilized onto said substrate. The interaction may be by covalent  
25 or by non-covalent binding.

*Bridge* – a physical connection, or a template for an electrical connector between two spaced electrodes.

*Nucleic acid fiber* - A specific example of a bridge composed of a sequence of nucleotides, which may be ribonucleotides, deoxyribo-nucleotides, other ribo nucleotide derivatives, a variety of synthetic, i.e. non naturally occurring nucleotides, as well as any combination of the above. The nucleic acid fiber  
5 may be single-stranded, double-stranded or multi-stranded, or any combination of the above.

*Functionalized bridge* - A bridge which conducts electric current either due to the inherent properties of its components or due to deposition of a  
10 substance or particles which impart conductivity onto the bridge. Such substances or particles may be bound to the bridge by a variety of interactions (e.g. may be chemically deposited on the bridge, may be complexed thereon by a variety of chemical interactions, may be associated with the bridge by electrostatic or hydrophobic interactions, etc.). The substances or particles  
15 may be bound to the bridge based on the general chemical properties of the fiber or may be bound to the bridge in a sequence specific manner. Examples of such substances or particles are: metals, metal colloids, conductive polymers, etc. A functionalized bridge may at times also be referred to herein as a "wire".

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*Functionalization* - the process of functionalizing the bridge.

*Electrode* - A conducting substrate, which may be made of metal or of any other conducting material or coated by metal or other conducting material,  
25 which serves for connection of the recognition or binding moiety to external electronic or electric components or circuitry, thus serving as an input/output (I/O) interface with an external component or circuitry.



*External circuitry, external component* - An electronic or electric circuitry or an electronic or electric component, situated electrically external to the electrodes and typically comprises prior art electric or electronic components, including standard solid-state microelectronic components.

5

*Linker* - An agent (molecule, complex of molecules, supramolecular structure, macromolecule, aggregate, colloid particle, molecular clusters, etc.) that acts in providing a physical link between the recognition moiety and the electrode or a substrate, thus serving to attach the recognition moiety to the electrode or the substrate. The linkers may have chemical groups for covalent or non covalent anchoring, (e.g. complexation or sorption, etc.) to the electrode or substrate, on the one hand, and to the recognition moiety on the other hand. Examples of linkers are: nucleic acid binding proteins; synthetic molecules with a binding ability to a specific nucleic acid sequence; a short, single or multiple stranded nucleic acid sequence (e.g. an oligonucleotide), e.g. having a "sticky end" and being modified at its other end, to allow it binding to the electrode; and non-biological molecules like derivatized alkyl silanes, etc.

15  
20 *Sample* - A medium which is to be tested for the presence of the target therein. Typically is a fluid obtained from a biological source, such as blood, urine, milk, food suspension, etc.

*Assaying (assay)* - a term referring collectively to both qualitative and/or quantitative determination of the target in a sample.

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*Assay device* - a device for use in the assaying of the target.

## SUMMARY OF THE INVENTION

The present invention is based on a new concept for detecting a target in a sample. The invention makes use of an assay device which comprises one or more sets of electrodes physically separated from one another. Each set of electrodes forms, together with an immobilized recognition moiety an assay set. The assay device is contacted with a sample and in the presence of the target in the sample, a bridge forms between different electrodes of an assay set. At times, the formation of the bridge gives rise to electric conductance between electrodes of an assay set. At other times, in order to yield electric conductance, an additional functionalization step of the bridge is required. Based on measurement of electric current or conductance, the presence of the target in the sample can be determined. In other words, in the presence of the target in the sample, an electrically conducting path is created between the electrodes and then is detected by performing electrical measurements. At minimum, the assay gives a qualitative result of the presence of the target in the sample, namely a "yes" or "no" answer; in other cases, the potential/current relationships can serve as a gauge for the concentration of the target in the sample.

The present invention thus provides, by a first of its aspects, an assay device for detection of a target in a sample, comprising one or more assay sets of electrodes, each assay set consisting of two or more electrodes and recognition moieties capable of specific binding to the target, the arrangement being such that binding of the target to the recognition moiety permits the formation of a functional bridge between at least two electrodes of an assay set for establishing electric contact between the at least two electrodes.

By another of its aspects the present invention provides a system for assaying of a target in a sample, which comprises at least one of the above devices and an external electric or electronic circuitry for

determining electric conductance between at least two electrodes of an assay set. The circuitry is thus capable of creating a potential difference between electrodes of the assay set, and measuring electric current which flows between different electrodes of an assay set.

5           The same or other electrodes may be used to create an electric field for directing a charged target to the recognition moiety. Thus, in accordance with an embodiment of the invention the electric or electronic circuitry is adapted for creating of such an electric field either through the same or different electrodes than the measuring electrodes. The electric  
10 field may also be of assistance in the step of forming a bridge between electrode of an assay set.

By yet a further aspect the present invention provides a method for assaying a target in a sample, comprising:

- (a) providing said assay device;
- 15 (b) contacting said assay device with said sample to form at least one bridge between at least two electrodes of an assay set;
- (c) providing conditions or treating the device to allow current flow across said bridge; and
- (d) determining electric conductance between different electrodes  
20 of an assay set, conductance above control level indicating the presence of said target in the sample. Optionally, the determination of conductance can also be made after different times of incubation of the assay device with the sample and determining concentration of the target from the time-conductance relationship.

25           The term "*determination*" or "*determine*" should be understood as referring collectively both to a qualitative measurement in order to deform in formation of a bridge (to achieve a "yes" or "no" answer) as well as to a quantitative measurement intended also to determine the extent of bridging.

In accordance with another aspect of the invention there is provides a reagent system, for use within the framework of some embodiments of the above methods, in order to functionalize the bridge formed between different electrodes of an assay set. The functionalization  
5 may, for example, be by depositing a conductive material, e.g. metal, on the bridge, and the reagent system will comprise the needed reagents for such a deposition.

The invention still further provides a kit useful in carrying out the above method, comprising said reagent system and an assay device and  
10 optionally also the external circuitry required for electric measurements.

The assay device comprises one or more assay sets each with at least two electrodes and a recognition moiety. In accordance with one embodiment, at least one electrode, and preferably two, of each assay set, have a recognition moiety immobilized thereon. Other electrodes of each  
15 assay set may have a binding moiety (namely a moiety which can bind a target in a semi specific or non specific manner) immobilized thereon or having surface properties such which allow non specific binding of the target thereto. Where recognition moiety is immobilized on at least two electrodes of an assay set, these recognition units may be the same or may  
20 be different (although binding to the same target). For example, one recognition moiety may be capable of specific binding to one epitope of the target and the other recognition moiety to another epitope. A specific example is the case of a nucleic acid sequence, where one recognition moiety comprises a sequence complementary to that of one portion of the  
25 target nucleic acid sequence and the other recognition moiety comprises a sequence complementary to another portion of the target nucleic acid sequence.

In accordance with another embodiment, the recognition moiety is immobilized on a carrier matrix which is other than the

electrodes. For example, the carrier matrix may be situated between electrodes of an assay set such that binding of the target thereto yields a formation of a bridge between at least two electrodes.

According to an embodiment of the invention, the target by  
5 itself has an electric conductivity and accordingly can such provide for the electric continuity between two electrodes. Such a target is for example a cell or another membranous structure.

In accordance with another embodiment, the target is not electrically conducting. At times, an *a priori*, non-conducting target need to  
10 be functionalized to become conducting, and participate in the current-conducting pathway (namely forms a building block of a functionalized bridge). Occasionally, however, the target by itself, although holding some or all of the bridge components, does not participate in the current-conducting pathway.

15 The formation of a functionalized bridge when performing the assay, indicates the presence of the target in the sample. It will obviously be clear to the artisan, that a small degree of electric conductance, and hence electric current when a potential difference exists between the electrode, may exist between electrodes of an assay set also under control conditions  
20 (e.g. under conditions identical to the assay conditions without a target in the sample). Thus whenever the existence of currents is discussed herein, it should be understood that this means current which is larger than under control conditions. While the assay may be limited to detection of the target in an all-or-none fashion (to give a "yes" or "no" answer), according to  
25 some embodiments of the invention, the assay may also be performed for the purpose of quantitative determination of the content of the target in the sample, e.g. determining its quantity. This may be achieved, for example, by performing the assay under conditions wherein the extent of bridging, e.g. the number of bridges formed in a given time period, depends on the

concentration of the target in the sample. For example, many recognition moieties may be immobilized on electrodes of an assay set and thus the number of those which bind to target, and hence the number of bridges which form, depends on the concentration of the target in a sample, possibly also dependent on the target quantity in the sample. The concentration may also be determined by employing a plurality of assay sets, all designed for the same target. In such a case, the number of the assay sets where functionalized bridges formed, out of the total number of assay sets, may provide an indication of concentration.

10           The concentration of the target in a sample may also at times be determined based on development of electric conductivity as a function of time. Such an assay, specifically several devices will be used, each incubated with a sample for a different period of time.

15           A device in accordance with the invention may comprise one or a plurality of assay sets. In case of a plurality of assay sets all may have the same target specificity (namely their recognition moiety will bind the same target) in which case, as pointed out above, the extent of bridging between electrodes in different assay sets may then serve as a quantitative measure for the concentration of the target in a sample. Alternatively, each assay set or a group of assay sets may have a different target specificity, in which case the device may be used in a multiplexing assay for assaying a panel of different target entities (e.g. different nucleic acid sequences). For example, each assay set or a group of assay sets may be specific for a different nucleotide sequence. Such a device may be useful in a variety of multiplexing diagnostic applications, i.e. simultaneous detection of a number of targets, detection of an unknown target which is then characterized by its binding (and hence bridge formation) to one of the recognition moieties in the different assay sets (e.g. randomly prepared nucleic acid sequences, each forming the recognition moiety in a different assay set). A specific example is the case of high

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throughput assays, for example, such aimed at finding a molecule, e.g. peptide, which specifically binds to another molecule, e.g. a receptor; assays for sequencing of unknown nucleotide strands (e.g. genome sequences); etc. In the case of a sequencing assay, a plurality of different sequences may be  
5 attached by linkers to electrodes of different assay sets and then a bridge formed in a specific assay set (where the specific nucleic acid sequence serves as the recognition moiety) identifies the specific sequence.

The target may be any one of a wide variety of entities including proteins, nucleic acid sequences, peptides, organic molecules, large  
10 macromolecular complexes, cells, cell membranes, viral particles, and many others. In case of a large entity such as a cell, cell membrane, viral particle, macromolecular complex, etc., the entire large entity, or only the portion thereof binding to the recognition moiety may be regarded as the target. For example, in the case of microorganism, the entire microorganism may be  
15 regarded as a target, or at times, where the recognition moiety binds to a specific antigen on the surface of the cell, such an antigen may then be regarded as the target.

Cell membranes and various macromolecules or macromolecular complexes may be inherently conductive. In such a case, the  
20 recognition/binding moieties may be *a priori* conductive, and thus upon binding to the target and the formation of the bridge, electric connectivity between the electrodes of the assay set is established. In other embodiments (e.g. where the target is a nucleic acid sequence, a protein, etc.) in order to establish electric conductivity between the two electrodes of the pair, a  
25 functionalization step may be required.

Depending on the nature of the target, the recognition moiety may be a single-stranded nucleic acid sequence or a double-stranded sequence with a sticky end, an antibody, a receptor, a lectin, a sugar, an antigen, etc. The recognition moiety and the target are thus members of an affinity group:

one member of the affinity group is the target, the other member of the group serves as the recognition moiety.

The recognition moiety may be immobilized on the electrode or on a non-electrode substrate, by the use of linkers which may be selected from  
5 a wide variety of molecules capable of attachment to a solid substrate on the one hand, and covalently binding or complexing to the recognition moiety on the other hand. Examples are a variety of sulfur-containing molecules which can, through their sulfur-containing moiety, be associated with a metal substrate such as gold, silver, platinum, etc. Such a linker may be covalently  
10 bound to the recognition moiety, or may be complexed thereto.

The measurement of electric conductance may be performed directly, by measuring current-potential relationship, or by performing other measurements indicative of the passage of current through the bridge. For example, the bridge may be functionalized in a manner that in addition to  
15 conducting current, it also emits light, in which case the electric connectivity of the bridge may be determined according to light emission.

Substances or particles which functionalize the bridge to render it conductive may be bound to the bridge based on the general (overall) properties of the bridge and based on the properties of the entities  
20 constituting the bridge. This may be achieved, for example, by chemically depositing an electrically conducting substance, e.g. metal onto the bridge. Alternatively, substances or particles may also be bound to the bridge in a sequence or domain-specific manner. Sequence or domain-specific deposition of substances at different bridge portions may be performed in a  
25 number of different ways. For example, a moiety capable of specifically binding to the bridge, for example, an antigen, *a priori* bound to a certain conductive substance or particle such as a metal ion, a metal colloid or a conductive polymer, may be made to bind to the bridge at a specific region recognized by it. Similarly, it is possible also to bind different types of



substances or particles, in a sequence or domain-specific manner, for example to a multi-stranded (e.g. double-stranded) nucleic acid fiber. This may be achieved, for example, by the use of a sequence-specific complexing agent which identifies and binds to a specific site of a double-stranded nucleic acid  
5 fiber. The complexing agent may be an oligonucleotide, forming with a double-stranded sequence, a triple-stranded structure; a protein recognizing a specific double-stranded domain; and many others.

By sequence or domain-specific binding, different types of substances may be bound to different portions of a given bridge.

10 The invention will now be illustrated in the following detailed description with occasional references to the annexed drawings. As will be appreciated, the description below is exemplary and should not be construed as limiting the scope of the invention as defined in the appended claims.

## 15 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic illustration of an assay device in a manner of performing the assay in accordance with an embodiment of the invention;

Fig. 2 is a schematic illustration of an assay device in a manner for performing the method in accordance with an embodiment of the invention;

20 Figs. 3A-3E show a different combination of recognition moieties, immobilized on at least one electrode of an assay device for the detection of target entities, in accordance with several different embodiments of the invention;

25 Figs. 4A, 4B and 4C are schematic illustrations of three embodiments of the invention where the recognition moiety is immobilized on a support member which is other than an electrode;

Fig. 5 is a schematic illustration of an assay device and the performance of a method in accordance with an embodiment of the invention, involving functionalization of the bridge.

Fig. 6 is a schematic illustration of an embodiment of the invention where the concentration of the target can be determined:

Fig. 7 is a schematic illustration of another embodiment of the invention for determining concentration of the target in the sample.

5 Fig. 8 is a schematic illustration of a multiplexing embodiment of the invention for detection of a variety of different target entities;

Fig. 9 is a schematic illustrating of an embodiment of the invention where each two adjacent assay sets share an electrode:

Fig. 10 illustrates an assay device and method for the detection of a  
10 DNA sequence in a sample;

Fig. 11 shows two exemplary current-voltage relationship of a functionalized bridge formed after metal deposition on a bridge-forming target as illustrated in Fig. 10;

Fig. 12 illustrates an assay device and method for the detection of a  
15 DNA sequence in a sample where the bridge is functionalized by deposition of poly-*p*-phenylene vinylene (PPV);

Fig. 13 illustrates another embodiment of functionalizing a nucleic acid bridge;

Fig. 14 shows an embodiment of the invention for assaying of an  
20 antigen;

Fig. 15 illustrates an embodiment of immobilization of oligonucleotide recognition moieties onto the electrodes;

Fig. 16 shows a scheme for synthesizing an oligonucleotide, as described in Example 1(a);

25 Fig. 17 shows a fluorescently labeled  $\lambda$ -DNA bridge stretched between two gold electrodes (dark strips) 12  $\mu\text{m}$  apart;

Fig. 18 shows atomic force microscope (AFM) images of a DNA bridge coated by silver connecting two gold electrodes 12  $\mu\text{m}$  apart 1.5  $\mu\text{m}$  and field size;

Fig. 19 is two terminal I-V curves of a DNA bridge coated by silver prepared according to Example 8. The arrows indicate the voltage scan direction. The solid-line curves are repeated scans and demonstrate the stability of the samples. Note the different asymmetry in the I-V curves  
5 corresponding to the two scanning directions;

Fig. 20 shows the I-V curves of a different silver wire in which the silver growth was more extensive than in Fig. 19. The more extensive silver growth resulted in a smaller current plateau, on the order of 0.5V, and a lower resistance (13M $\Omega$  vs. 30 M $\Omega$  in Fig. 17). By driving large currents  
10 through the wire, the plateau has been eliminated to give an ohmic behavior (dashed line), over the whole measurement range;

Fig. 21 shows a schematic representation of the steps of performing a detection assay for the presence of a nucleic acid sequence in a sample:  
and

15 Fig. 22 shows a device comprising an array of pairs of electrodes. The device is designed for a multiplex detection of a target or a plurality of targets in a sample.

## DETAILED DESCRIPTION OF THE INVENTION

20 Reference is first being made to Fig. 1, illustrating an assay device 102 consisting of a single assay set with two electrodes 104 and 106 connected to one electric or an electronic circuitry 108. Immobilized on electrodes 104 and 106 are recognition moieties 110 and 112. In (a) there is no electric contact between electrodes 104 and 106.

25 When the assay device 102 is contacted with a sample comprising a target 114, bridge 116 forms between the two electrodes 104 and 106. Under appropriate conditions or by a subsequent functionalization step (see below) the bridge is conductive and current can flow through the bridge between the two terminals of module 108, as represented by the

B-directional arrow 118 (b). In the embodiment shown in Fig. 1, the assay set comprises two electrodes.

In Fig. 2, a different embodiment is illustrated, where an assay set 122 consists of three electrodes 124, 126 and 128 having three different recognition moieties 130, 132 and 134, respectively, immobilized thereon. These three electrodes are connected to an electric or electronic control module 136. Each of the immobilized recognition moieties 130, 132 and 134, can bind to a different moiety in the target 138.

When the device (a) is contacted with the sample containing the target 138, target entities can bind to the different electrodes in one of the manners illustrated schematically under (b1), (b2) and (b3). Under appropriate conditions or following subsequent functionalization step, current can flow through the formed bridges as illustrated by arrows 140, 142 and 144. Measurement of current flow in either one of the formed circuits, namely, between terminals 146-148, 146-150 or 148-156 of module 136, yield information on the target.

Figs. 3A-3E show different configurations of assay sets in accordance with different embodiments of the invention. In assay set 160, shown in Fig. 3A, each of electrodes 162 and 164 has immobilized thereon a recognition moiety 166 and 168, respectively. For example, recognition moieties 166, 168 may be oligonucleotides complementary to terminal sequences in a target nucleic acid sequence 170.

In Fig. 3B, of the two electrodes 174 and 176 of assay set 172, only the former has immobilized thereon a recognition moiety 175, e.g. an oligonucleotide which is complementary to terminal sequence of target nucleic acid molecule 178. Target nucleic acid molecule 178 binds specifically to recognition moiety 175 and then non specifically or semi specifically to electrode 176.

Fig. 3C, illustrates an assay set 180 which has two electrodes 182 and 184 with immobilized recognition moieties 186 and 188, which in this specific embodiment are different antibodies, recognizing different antigenic epitopes of a target 190.

5           Assay set 192 shown in Fig. 3D, has two electrodes 194 and 196 each of which has immobilized thereon a relatively long oligonucleotide 198 and 200, respectively, the terminal sequence of which constitutes the recognition moiety for the target, in this specific case a short oligonucleotide 202. Target oligonucleotide 202 thus brings together two  
10 oligonucleotides 198 and 200, without participating in the bridge itself.

          Assay set 210 shown in Fig. 3E consists of two electrodes 212 and 214 having each a recognition moiety 216 and 218, respectively, immobilized thereon. Each of these recognition moieties binds to an epitope on the external surface of a cell 220, which once bound to the recognition  
15 moiety bridges the two electrodes 212 and 214.

          In all the embodiments shown in Figs. 3A-3E, a recognition moiety is immobilized on at least one electrode of an assay set. Against the case of the embodiments of the assay sets 222 and 234 and 244 shown in Figs. 4A-4C, no recognition moiety is immobilized on the respective  
20 electrodes 224 and 226, 236 and 238 and 245 and 246. Rather, in this case, each of the assay sets 222, 234 and 244 have a substrate member 228, 240 and 247, respectively, which are other than the electrodes, on which the respective recognition moieties 230, 242' and 242" and 248 are bound. In the case of assay set 222, immobilized on member 228 is a single recognition  
25 moiety 230, which in this specific embodiment is an antibody directed to an epitope of target 232 (the target may be a nucleic acid sequence, a polymer, a polypeptide, etc.). In the case of assay set 234, member 240 has immobilized thereon two oligonucleotide substrates 242' and 242" which are complementary to portions of target nucleic acid sequence 244. In both cases,

after binding to the recognition moieties, a bridge between the two electrodes of the assay sets forms by a non-specific or semi-specific binding or association. In the case of assay set 244, after the oligonucleotide target 249 binds to the recognition moiety 248, it serves a template for synthesis of other  
5 nucleic acid sequences, and this synthesis eventually forms a bridge 249A between the two electrodes 245 and 246.

Each of the assay sets shown in Fig. 4A-4C, have two electrodes. It will readily be understood that the illustrated embodiments apply also to the case of more than two electrodes in each assay set.

10 Fig. 5 is a schematic illustration of a manner of performance of the method in accordance with an embodiment of the invention involving a functionalization step. Assay set 250 (a) is contacted with a target 251 to form a bridge 252 (b). After the functionalization step, a functionalized bridge or wire 253 is formed (c).

15 Fig. 6 shows the manner of determining concentration of a target in a sample in accordance with an embodiment of the invention. Each of electrodes 258 and 259 of assay set 256, has immobilized thereon a plurality of recognition moieties 260 and 261, respectively. After contact with a target 264, one or more bridges between the electrodes form. In a case of  
20 low concentration of the target 264 (b1), a small number of bridges forms in a given time period (illustrated here by a single bridge 266) and in the case of a high concentration (b2), a large number of bridges, illustrated here by six bridges 268 formed within the same time period. It is clear that the measured resistance during the same time period is lower in the case of a high  
25 concentration as compared to a low concentration. This difference in the potential/current relationship can thus serve as a measure (after proper calibration) of concentration of the target 264 in a sample.

Fig. 7 is a schematic illustration of a device 270 having a plurality of assay sets 271, each comprising two electrodes and having the

same recognition moieties immobilized thereon. When the naive assay device 270 is contacted, for a given time period, with a target 272, in the case of a low concentration, bridging between the different electrodes of each assay set forms only in a few assay sets. whereas in the case of a high concentration (b2) bridges form in many (at times all) assay sets. Counting the number of units where current is detected, indicates the concentration of the target 272 in the sample.

Fig. 8 illustrates a multiplexing embodiment, where each assay set or a group of assay sets is designed for binding a different target. This allows a diagnostic assay for the simultaneous detection of a number of target entities in a sample. In the illustration, each of the assay sets or group of assay sets 282A-282D, have a different target specificity, as illustrated by the different shapes of the immobilized recognition moieties 283A-283D respectively. When the assay device 280 is contacted with a sample, comprising, for example, target entities 284A and 284D, bridges form only in assay sets 282A and 282D which then serves as an indication of the presence of the respective targets in the sample.

In all assay devices illustrated in Figs. 1-8, each assay set has its own electrodes. At times, however, two or more assay sets may have common electrodes. In a sense, the assay set 122 of Fig. 2 can be viewed as three assay sets with each two electrodes defining a different assay set. An illustration of an embodiment where each two adjacent assay sets share an electrode can be seen in Fig. 9. As illustrated in (a) of this figure, the assay device 289 has a plurality of assay sets of which three, 290AB, 290BC and 290CD, can be seen. Seen in this illustration are four consecutive electrodes, 291A, 291B, 291C and 291D having immobilized thereon respective recognition moieties 292A, 292B, 292C and 292D. Each two adjacent electrodes define one of the assay sets, e.g. electrodes 291A and 291B defining assay set 290AB, with each two adjacent assay sets sharing a common electrode: assay sets 290AB

and 290BC sharing electrode 291B and assay sets 290BC and 290CD sharing electrode 291C.

The recognition moieties 292A, 292B, 292C and 292D bind to respective epitopes 294A, 294B, 294C and 294D. Thus assay set 290AB will  
5 be specific for target 293AB having epitopes 294A and 294B, assay set 290BC will be specific for target 293BC having epitopes 294B and 294C and assay set 290CD will be specific for target 293CD having epitopes 294C and 294D. Consequently, when contacted with a sample, a bridge will form in an assay set, depending on the type of target in the sample as illustrated under  
10 (b1), (b2) and (b3).

Reference is now being made to Fig. 10, illustrating an assay device and method for detection of a specific target DNA sequence 310 in a sample. The detection is carried by formation of a bridge 312 between two electrodes 300, which is then typically coated by metals such as gold.  
15 platinum, silver, etc. to eventually yield a functionalized bridge 320. For the formation of the assay device, electrodes 300 may be first treated in a manner to facilitate subsequent binding of molecules 302 and 304. For this purpose the electrodes may be first wetted separately with a solution containing either molecules 302 or 304, each being a single-stranded oligonucleotide, which  
20 serves as a recognition moiety ("*Oligo A*" and "*Oligo B*", respectively), derivatized by a disulfide group for attachment of molecules 302 and 304 to the electrodes. Oligonucleotides 302 and 304 are each complementary to one of the two terminal sequences of the target DNA sequence 310. When these recognition moieties are deposited on electrodes 300, under appropriate  
25 conditions, the disulfide group bind to the electrodes 300, to form recognition moieties.

For detection of the target in a sample, the assay device is contacted with a sample suspected of comprising a target nucleotide sequence, in this specific example a single or a double-stranded DNA sequence 310. The



sample may be a blood sample, a food sample, a water sample, etc. In the case where the target is a double-stranded sequence, the target in the sample may be treated to form single-stranded sticky ends at the terminals of a double-stranded target DNA 310. These sticky ends are complementary to the sequences of the oligonucleotides in recognition moieties 306 and 308. Electrodes 300 are spaced from one another at a distance which should not exceed the combined length of the target DNA 310 and the recognition moieties 306 and 308. When the electrodes are contacted with a sample comprising target DNA sequence 310, the target terminal ends connect to their complementary oligonucleotide sequence in the recognition moieties 306 and 308 to form a bridge 312 between the two electrodes 300 (step (b)). In the case of targets which are double-stranded, following hybridization, the binding of the recognition moieties 306 and 308 to the target nucleic acid sequence 310 may be strengthened by ligating the nicks to form covalent binding.

At times, particularly where sequence 310 is long, it may not be practical to rely on diffusion for hybridization of the target 310 to the recognition moieties 306 and 308. In such a case, the target 310 may be made to connect to one electrode and then, by a directional stream of fluid from the first electrode to the second, or by applying an electric field the nucleic acid bridge is made to extend so that its end reaches the second electrode.

It is also clear that in order to avoid folding of the nucleic acid molecules and to ensure proper binding, appropriate solutions may at times be needed. In addition, after hybridization, rinsing may at times be required in order to remove unbound nucleic acid strands.

The functionalization step of the bridge, for the purpose of establishing electrical conduction, begins, according to the specifically illustrated embodiment, by an ion exchange step involving exposure of the nucleic acid fiber to an alkaline solution comprising silver ions ( $\text{Ag}^+$ ). The

silver ions replace the sodium ions or other ions normally associated with the nucleic acid molecule and complex with the negatively charged nucleic acid sequences (step (c)). (It should be noted that  $\text{Ag}^+$  ions may also be made to bind to nucleic acid molecules in various other ways in particular by  
5 intercalation). These steps give rise to a nucleic acid sequence **314** loaded with silver ions **316**. It should be noted, that rather than silver ions, a wide variety of other ions can also be used, including for example, cobalt, copper, nickel, iron, gold, etc. Furthermore, metal aggregates, semiconductor particles, complexes or clusters, e.g. colloidal gold, colloidal silver, gold  
10 clusters, etc., may also be deposited on the nucleic acid sequence via a variety of different interactions. Conductive oligomers and polymers may also serve to functionalize the nucleic acid bridge.

At a next step (step (d)), the sequence is exposed to a reducing agent, e.g. hydroquinone, or to an electromagnetic radiation, to reduce the  
15 metal ions *in situ* into metallic silver which forms nucleation sites **318**. In a different embodiment metal nucleation centers may be formed by attaching a host of colloids or clusters to the DNA bridge in a sequence specific or non-specific manner. After rinsing a reagent solution comprising metal ions and a reducing agent, e.g. hydroquinone under acidic conditions, is added.  
20 Under these conditions, the ions are converted to metal only at nucleation sites and consequently the nucleation centers grow and merge with each other to form a conductive functionalized bridge **320** (step (e)).

The so formed functionalized bridge **320** may be subjected to a variety of post fabrication treatments, which may include, for example,  
25 thermal treatment intended to increase the bridge's thickness and homogeneity; passivation treatment for the purpose of forming an electrically insulating layer around the bridge, e.g. by exposure to alkane thiol; electrochemical or photochemical coating of the wire using polymers.

Fig. 11 illustrates two exemplary current-voltage relationships

of a functionalized bridge formed by the procedure illustrated in Fig. 9. Different current-voltage relationships may be obtained depending on the type of conductor and the functionalization process, etc.

Reference is now being made to Fig. 12. illustrating a device and method of the invention wherein functionalization is carried out by depositing a conductive polymer PPV, (poly-*p*-phenylene vinylene). Electrodes 400, may be the same as electrodes 300 shown in Fig. 10. The first two steps of the detection method (steps (a) and (b)) may be identical to the corresponding steps in Fig. 10 (identical components have been given a reference numeral with the same last two digits as the corresponding components in Fig. 10: e.g. 402 is the same as 302, 404 as 304, etc.). The formed bridge 412 may be strengthened, similarly as above, by covalent binding of bridge 410 to the recognition moieties 406 and 408 to yield a bridge 414 connecting the two electrodes (step (c)).

A solution comprising pre-PPV molecules 416 is then contacted with bridge 414. By the virtue of being positively charged, pre-PPV 416 becomes complexed with the negatively charged DNA bridge 414 (step (d)). At a next step, conjugation is induced by removal of tetrahydrothiophene groups and hydrochloric acid from each repeat unit, yielding a luminescent PPV bridge (step (e)). This component is suitable for optical detection. Alternatively, the PPV may be doped with agents which either cause electron deficiency (holes) or give rise to extra electrons and thus converted into a conducting polyer. Doping may be performed by many known methods e.g. exposure to H<sub>2</sub>SO<sub>4</sub> vapor. The extent of doping determines the conductivity of the PPVwire.

Many other conductive polymers may be used instead or in addition to PPV in accordance with the invention. This includes a variety of polymers with positively charged side groups as well as polymers with positively charged groups in the backbone, polymers with recognition groups

capable of binding to nucleic acid fibers or polymers that complex with DNA. In addition in a similar manner, *mutatis mutandis*, other types of conducting substances (n-type or p-type) may be bound to the fiber.

Yet another embodiment of the invention is illustrated in Fig. 13. In this figure, identical components to those of Fig. 12 are shown by numerals having the same two last digits. Nucleotide bridge **514** formed by the recognition process described with reference to Fig. 12, is exposed to a solution containing a monomer oligomer, or polymer **516**. As a result, ion exchange or other complexing occurs, leaving the nucleotide bridge **514** loaded with substance **516**. Polymerization step is then applied to form the functionalized bridge **517** connecting the electrodes concerning detection of an antigen by use of an antibody. A doping process renders the functionalized bridge conductive.

## 15 EXAMPLES

### Example 1: Preparation of Linkers between the Electrodes and the Recognition moiety

#### (a) Disulfide based linkers:

20 Controlled pore glass (CPG) derivatized with a disulfide group is used for the synthesis (starting from its 3' side) of an oligonucleotide having a free 5' site which serves as the recognition moiety. The oligonucleotide is prepared using a conventional DNA synthesizer (see scheme in Fig. 16).

#### (b) Thiol-based linkers:

25 Linkers are being prepared according to (a) above and the disulfide bond is cleaved to obtain a free thiol.

(c) Biotin-streptavidin complex based linkers:

Biotin moiety is attached to an oligonucleotide having a specific sequence, as known *per se* which will serve as the recognition moiety. The biotin-oligonucleotide is coupled via a streptavidin molecule to  
5 another molecule containing a biotin moiety at one side (see also Fig. 15) and a thiol or disulfide group on the other side.

(d) Repressor based linkers:

A nucleic acid binding protein, such as the *lac* repressor, is covalently attached to a thiol group. A DNA sequence, serving as the  
10 recognition moiety is synthesized containing also the specific sequence to which the repressor binds. The DNA is coupled to the repressor through said specific sequence.

(e) Thiophosphate based linkers:

The construction (starting from its 3' side) of an oligonucleotide, serving as a recognition moiety, is carried out using a conventional  
15 DNA synthesizer wherein thiophosphates containing-nucleotides are used instead of standard nucleotides.

(f) Artificial site specific based linkers:

A synthetic site-specific moiety such as, for example  
20  $\text{Rh(Phen)}_2\text{Phi}$ , known to bind 5'-pyr-pyr-pur-3' sequence (pyr = pyrimidine, pur = purine), is covalently coupled to a thiol group.

**Example 2: Attachment of the linker to an electrode**

(a) Micropipette Wetting:

25 Electrodes are exposed to solutions of the appropriate linkers, for example, by employing pipettes or micropipettes or by any liquid dispensers. Such liquid dispensers may be fixed onto a manipulator that may be computer controlled. Different types of linkers can be deposited on each

electrode. Additionally, different types of linkers can be deposited simultaneously or sequentially on different electrodes.

(b) Jet printing:

5 Ink-jet like printing techniques are used for the selective exposure of different electrodes to different linkers. By utilizing such a technique, it is possible to attain high precision, resolution, and to increase rates of production, facilitating large scale production.

Electrode-linker synthesis:

10 (c1) Using selective masking techniques:

The well developed technology used for synthesizing DNA sequences may be harnessed for the *ab-initio* preparation of a complex electrode-linker array. For example: a substrate composed of an assay set of electrodes on an inert substrate is partially coated with an inert  
15 coating yielding two types of electrodes: coated electrodes (A) and uncoated electrodes (B). The substrate is exposed to a solution of a thiol linker linked to a nucleic acid sequence serving as a seed for DNA synthesis of a sequence which will eventually serve as the recognition moiety. Due to the inert coating, only the uncoated B electrodes react  
20 with the thiol. Using standard DNA synthesizing techniques, a pre-defined sequence, being the recognition moiety, is produced on the B electrodes. The substrate is then rinsed and the masked electrodes are uncovered followed by the selective coating of B electrode. This procedure allows the production of two types of electrodes differing one  
25 from the other by the type of recognition moieties bound thereto. The same technique with some additional steps (several steps of masking and unmasking) allows the fabrication of various substrates having many different electrodes with different recognition moieties bound thereto.

(c2) Using photodeprotection techniques:

This approach involves the utilization of photolabile groups for the protection of the start point of DNA synthesis. Inactivated start point groups are unable to react with nucleotides. Using selective irradiation by means of a mask and/or a light conductor and/or any other addressable light source, the activation of different selected electrodes is achieved by the photoremoval of protecting groups from the DNA synthesis seeds on selected electrodes.

(c3) Using blockers:

Using the masking technique ((c1) above) an assay set of electrodes is prepared for oligonucleotide synthesis. Once a DNA sequence, which serves as the recognition moiety, is completed on one assay set of electrodes, a terminating group (blocker) is attached to the oligonucleotide ensuring their inertness. Other sequences can be further synthesized on different electrodes that are prepared according to the previous step but become active according to this step. It should be noted that the assay set of linkers constructed in the previous step is not affected due to the blockers attached to their end points.

(c4) Electrode printing:

Recognition moieties are attached to conducting beads such as gold colloids. The colloids are then dispersed in a controllable manner to form conducting metal pads with linkers and recognition moieties attached thereto. Dispensing may be achieved by the different techniques outlined above or by any conventional technique. The electrode may be made conductive, *a priori*, or at the end of its preparation.

The above techniques may be used alone or in any combination with other techniques.

**Example 3: Connection of a nucleic acid fiber to an electrode or a carrying substrate**

5            Attachment of a nucleic acid fiber to a substrate, which may be the electrode or the substrate on which the device is formed is carried out using DNA binding proteins. For example, repressors from a bacterial origin (*lac* – repressor or  $\lambda$  repressor) which can bind to both the substrate (for example a plastic substrate) and the DNA thus joining the two. Such  
10 connection may be later functionalized, together with the nucleic acid fiber to render the bridge conductive, for example, when it connects two electrodes. Alternatively such connection may merely serve to stabilize the bridge to the carrying substrate without participating in the electric functionality.

15   **Example 4: Preparation of an integrated circuit for detection purposes**

             The integrated circuit (IC) is composed of a substrate such as silicon, derivatized silicon, silica, derivatized silica, organic polymer or any other substance capable of acting as a support for the fabrication or mechanical fixation or stabilization of the functionalized bridge. The substrate  
20 may serve an electrical function.

             A typical example for IC preparation is described in the following:

**Example 5: Passivation of a glass substrate**

25            A glass substrate is immersed in fuming nitric acid (100%  $\text{HNO}_3$ ) for 10 min, rinsed with deionized (DI) water, then immersed in 1 N NaOH solution for an additional 10 min and rinsed with DI water. The cleaned glass is dried thoroughly, then immersed for c.a. 12 hrs in a solution of an alkyl trichlorosilane (octyl trichlorosilane, trimethyl trichlorosilane



etc.) in tetrachloroethane (1:5 v/v). The glass plate is then rinsed carefully several times with tetrachloroethane and isopropanol, then dried thoroughly.

**Example 6: Electrode fabrication**

5                Electrodes are fabricated according to one of the following routes: (i) Standard photo, electron, or x-ray lithography on the substrate and subsequent deposition of a conductive substance (e.g. metal). Alternatively, the conductive substance may be deposited first and patterned next. (ii) Electrode assembly onto the surface: Patterning of the glass surface using  
10 polyelectrolytes such as polyethyleimine, polyalcoholes, polyacids, polypyridines etc. or other ligating agents such as a thiol monolayer (fabricated from organic compounds containing thiol and silane moieties at opposite sites on the molecular skeleton) followed by the fixation of electrically conducting components such as Gold colloids enabling the  
15 assembly of conducting electrodes onto the substrate.

**Example 7: Electrical functionalization of a bridge composed of a nucleic acid affinity group - metal based conductive bridges**

20            (i)    The bridge made of two complementary nucleic acid sequences is exposed to a solution containing the appropriate metal ion, thus, ion exchange occurs at the phosphate groups of the nucleic acid skeleton exposed to the solution. Intercalation of ions inside the nucleic acid may also take place under certain conditions;  
25            (ii)    The ion exchanged nucleic acid complex is then reduced by a reducing agent such as hydroquinone or by exposure to electromagnetic radiation.

Cycles (i) and (ii) can be repeated in a sequential order until a conducting wire is achieved. Alternatively, the formation of conducting metal  
30 wire includes the following steps as stand-alone processes or in conjunction

with steps (i) and (ii) or combined with one or more of the following techniques.

(iii) The relevant part of the ion-exchanged bridge is exposed to a metastable mixture of the reducing agent and metal ions. Reduction takes place only at the surface of the metal clusters formed by steps (i) and (ii) thus, the gap between the metal clusters is bridged by the metal deposition process.

(iv) The ion exchanged nucleic acid sequence or the partially treated nucleic acid bridge is exposed to electrochemical processors, transforming the ions loaded on the nucleic acid polyelectrolyte into a metallic conductor. In addition, electrochemical processes along the nucleic acid molecule promote the vectorial growth of the metal wire along it.

(v) Photochemical deposition of the metal from its corresponding ions for the formation of the metallic wire.

(vi) Clusters or colloids are adsorbed onto the nucleic acid bridge using sequence selective components, for example, specific sequences which are capable of binding to specific sites on the nucleic acid sequence non-sequence-specific binding agents, e.g. polyelectrolytes undergoing electrostatic interactions with the DNA. These Clusters and/or colloids serve as catalysts for processes (iii)-(v) above.

(vii) Defects in granular wires fabricated by one or more of the above techniques may be annealed using diverse methods such as thermal annealing processes, electrodeposition, etc.

An example of the fabrication of a silver-functionalized bridge is as follows:

(i) A DNA fiber fixed on a substrate is exposed to a basic solution of silver ions (pH=10.5,  $\text{NH}_4\text{OH}$ , 0.1 M  $\text{AgNO}_3$ ). After the DNA polyelectrolyte is exchanged by the silver ions, the substrate is rinsed carefully with deionized water (DI) and dried.

(ii) The silver loaded DNA bridge fixed on a substrate is exposed to a basic solution of hydroquinone (0.05 M, pH = 5) as a reducing agent. Steps (i) and (ii) are repeated sequentially until an electrically conducting wire is formed.

5

Complementary processes:

(a) step (iii) is performed after one or more (i)+(ii) cycles.

(iii) The DNA fiber loaded with silver metal clusters (after cycles (i) and (ii) have been performed) and after final rinsing with DI water is exposed  
10 to an acidic solution of hydroquinone (citrate buffer, pH=3.5, 0.05 M hydroquinone) and  $\text{AgNO}_3$  (0.1 M). Cycle (iii) is terminated when the wire width attains the desired dimension. The process can be made light sensitive and thus can also be controlled by the illumination conditions.

(b) Electrochemical deposition for improved process:

15 (iv) In order to expedite and improve the metallic conductor, an electrochemical process is performed. For that purpose, pre-treatment with an alkane thiol is performed prior to the (i)+(ii) processes. This ensures the inertness of the metal electrodes against electrochemical metal deposition. After one or more of the (i)+(ii) cycles, the electrodes connected through the  
20 DNA-covered metal wire are connected to a current or bias controlled electrical source and the relevant part of the DNA fiber is exposed to a solution of the metal ion (different concentrations according to a specific protocol). The gaps between the conducting domains are filled via electrochemical metallic deposition.

25 (c) Photochemical deposition for an improved process:

(v) In order to improve the metallic conductor, a photochemical process is performed in a similar manner to the electrochemical process outlined above but using photochemical reaction as driving processes. For example, metalization of a DNA fiber may be obtained using an electron

donor (triethanolamine, oxalic acid, DTT etc.), a photosensitizer (Ru-polypyridine complexes, xanthene dyes semiconductor particles such as  $\text{TiO}_2$ , CdS etc.), an electron relay such as different bipyridinium salts and the relevant metal ion or metal complex. The photosensitizer transduces the absorbed light energy into a thermodynamic potential through electron transfer processes involving the electron donor and electron acceptor in any of the possible sequences. The reduced electron acceptor acts as an electron relay and charges the metal clusters/colloids with electrons. The charged clusters/colloids act as catalysts for the reduction of the metal ions thus inducing the growth of the metal conductor.

(d) Gold clusters and/or colloids as nucleation centers:

(vi) Instead of performing the first (i)+(ii) cycles, the relevant part of the DNA bridge is exposed to a solution of gold colloids pre-coated (partially) with cationic thiols (such as pyridinium alkane thiol). The Gold colloids are being adsorbed to the DNA skeleton by ion pairing and the growth of the wire is attained using one or more of the above techniques. Alternatively, the gold colloids may be attached by various means such as biotin-streptavidin binding to modified nucleotides, e.g. modified with biotin.

(e) Curing processes:

(vii) Defects in a granular wire obtained by one or a combination of the above techniques are annealed using diverse processes such as thermal annealing processes (hydrogen atmosphere (10%  $\text{H}_2$  in  $\text{N}_2$ ), 300C over several hours).

**Example 8: Detection of the presence of  $\lambda$ -DNA in a sample**

(a) Device preparation

The detection of  $\lambda$ -DNA relies on the fact that such a molecule possesses two 12-base sticky ends. Fig 10 outlines the fabrication of a device capable of detecting  $\lambda$ -DNA. A glass coverslip is first passivated against

spurious DNA binding. Subsequently, two parallel gold electrodes are deposited on the coverslip using standard microelectronic techniques. One gold electrode is then wetted with a micron size droplet of an aqueous solution containing a 12-base, specific sequence oligonucleotides, 5 derivatized with a disulfide group attached to their 3' side. Similarly, the second electrode is marked with a different oligonucleotide sequence. The two sequences (oligo A and B in the figure) are complementary to the  $\lambda$ -DNA sticky ends. After rinsing the device is ready for detection.

(b) Detection

10 A solution suspected of containing a 16  $\mu\text{m}$  long  $\lambda$ -DNA, having two 12-base sticky ends that are complementary to the oligonucleotides attached to the gold electrodes is made to flow normal to the electrodes. The flow is induced to stretch the DNA, allowing its hybridization with the two distance surface-bound oligonucleotides. In case the sample 15 contains  $\lambda$ -DNA molecules they bind and form a bridge connecting the electrodes. Fig. 17 depicts the results of such an experiment; a fluorescently-labeled  $\lambda$ -DNA bridging the two electrodes.

Two-terminal measurements performed on these samples prove that the stretched DNA molecule is practically an insulator with a resistance 20 higher than  $10^{13}\Omega$ . To detect the presence of DNA bridges they are first instilled with electrical functionality, by vectorially depositing silver metal along the DNA molecule. The three-step chemical deposition process (Fig. 10 (c)-(e)) is based on selective localization of silver ions along the DNA through  $\text{Ag}^+/\text{Na}^+$  ion exchange and formation of complexes between the silver and the 25 DNA molecules. After rinsing, the silver ion-exchanged -DNA complex is reduced using basic hydroquinone solution. This step results in the formation of nanometer size metallic silver aggregates bound to the DNA skeleton. These silver aggregates serve as spatially localized nucleation sites for subsequent growth of the wire. The ion-exchange process is highly selective

and restricted to the DNA only. The silver aggregates, bound to the DNA, are further "*developed*", much as in the standard photographic procedure, using an acidic mixture of hydroquinone and silver ions under low light conditions. Acidic solutions of hydroquinone and silver ions are metastable but  
5 spontaneous metal deposition is normally very slow. The presence of metal catalysts (such as the silver nucleation sites on the DNA), significantly accelerates the process. Under these experimental conditions, metal deposition therefore occurs only along the DNA skeleton, leaving the passivated glass practically clean of silver.

10 Atomic force microscope (AFM) images of a section of a 100 nm wide, 12 $\mu$ m long wire are presented in Fig. 14. As clearly seen, the wire comprises of 30-50 nm-diameter grains deposited along the DNA skeleton. Fig. 19 shows the I-V curves of the silver presented in Fig. 18. The length of the zero bias plateau in different wires can be tuned from zero volt to  
15 roughly 10 volts. The solid line in Fig. 20 depicts, for example, the I-V curve of a different wire in which the silver growth on the DNA was more extensive. As a result, the plateau can be eliminated to give an ohmic behavior (dashed line in Fig. 20).

This example proves that  $\lambda$ -DNA molecules can indeed be  
20 detected using the present invention.

#### **Example 9: Organic conjugated-polymer based conducting wires**

A schematic representation of the manner of production of organic conjugated polymers is shown in Fig. 12 step (a)-(b) are similar to  
25 those disclosed in Fig. 10.

(i) The relevant part of the bridge is exposed to a solution containing a cationic segment capable of forming a conjugated-polymer by a chemical transformation or a cationic non conjugated-polymer capable of undergoing conjugation by a chemical transformation or a cationic

conjugated-polymer. Thus, ion exchange process occurs at the phosphate groups of the DNA skeleton exposed to the solution.

- (ii) The ion exchanged DNA complex is treated according to the nature of the organic species that is bound to the polyanionic skeleton.
- 5 Electrical functionalization is achieved either by the former process or by a sequential doping process. Doping may be achieved via conventional redox processes, by protonation - deprotonation processes, by electrochemical means or by photochemical means. Additionally, sequence selective processes
- 10 conjugated-polymer based conducting wires can be utilized for the production of wires.

I. The fabrication of a PPV (poly-*p*-phenylene vinylene) conducting wire is as follows:

- (i) A DNA fiber fixed on a substrate (b) is exposed to a solution of
- 15 a pre-PPV water soluble polymer. After the DNA polyelectrolyte is exchanged by the pre-PPV polymer, the substrate is rinsed carefully and dried.

(ii) The pre-PPV polymer loaded DNA fiber fixed on the substrate is reacted in a vacuum oven (e.g 1e-6 bar, 300 C, 6hr.).

- (iii) The resulting luminescent PPV polymer is doped using conventional methods until displaying conductivity.
- 20

II. An alternative route for the fabrication of a PPV conductive wire is as follows:

- (i) A DNA bridge fixed on a substrate (Fig. 12(a) and 12(b) is exposed to a solution of a bis-(tetrahydrothiophenium)-*p*-xylilene dichloride
- 25 (Fig. 12(c). After the DNA polyelectrolyte is exchanged by the bis-(tetrahydrothiophenium)-*p*-xylilene dichloride, the substrate is rinsed carefully and dried.

(ii) The bis-(tetrahydrothiophenium)-*p*-xylilene dichloride loaded DNA sequences fixed on a substrate is polymerized in a basic solution to form a pre-PPV polymer attached to the DNA backbone (Fig. 12(d)).

(iii) The pre-PPV polymer loaded DNA sequences fixed on a  
5 substrate is reacted in a vacuum oven ( $1\text{e-}6$  bar, 300 C. 6hr.).

(iv) The resulting luminescent PPV polymer is doped using conventional methods until displaying desired conductivity.

III. The fabrication of a PANI (polyaniline) conducting wire is carried out as follows:

10 (i) A DNA bridge fixed on a substrate is exposed to a solution of an acid soluble PANI polymer. After the DNA polyelectrolyte is exchanged by PANI polymer, the substrate is rinsed carefully and dried.

(ii) The resulting PANI polymer is doped using conventional methods until displaying desired conductivity.

15 IV. An alternative route to the fabrication of a PANI conducting wire is as follows:

(i) A DNA bridge fixed on a substrate is exposed to a solution of anilinium ions. After the DNA polyelectrolyte is exchanged by the anilinium ion, the substrate is rinsed carefully and dried.

20 (ii) The anilinium ions loaded on the DNA sequences are oxidized using a solution of an oxidizing agent such as peroxidisulphate ions, yielding a polyaniline polymer. The resulting PANI polymer is doped using conventional methods until displaying desired conductivity.

V. An alternative route to the fabrication of a PANI conducting  
25 wire is as follows:

(i) A DNA bridge fixed on a substrate is exposed to a solution of a short oligomers of PANI ( $>1$  repeat unit). After the DNA polyelectrolyte is exchanged by the PANI oligomer, the substrate is rinsed carefully and dried.



(ii) The PANI oligomer ions loaded on the DNA sequence are oxidized using a solution of an oxidizing agent such as peroxodisulphate ions, yielding a polyaniline polymer. The resulting PANI polymer is doped using conventional methods until displaying desired conductivity.

5

**Example 10: PPV functionalized fiber as a light source**

The process described in Example 9 may be followed up to and including step I(ii). The resulting PPV component is highly luminescent. Fabricating the PPV component between electrodes of appropriate work  
10 functions then forms an electroluminescent device.

**Example 11: Polymer supported recognition moiety for selective attachment of recognition moieties to electrodes**

15 **Specific Examples:**

N-(2-ethyl maleimido) pyrrole is attached to a 3'-thio modified specific sequence of monostranded oligonucleotide. Electro-oxidation of a solution containing the 3'-mercapto(N-(2-ethyl succine imido) pyrrole) oligonucleotide induces the formation of a polypyrrole coated electrode  
20 bearing specific sequence oligonucleotides. The polymer is deposited exclusively at the anode side allowing the selective coating of a plurality of electrodes simply by dipping the electrode assay set into a series of solutions containing the desired sequences each time using a different electrode as the anode. Since polypyrrole is a conductor in its doped state, electric  
25 connectivity of the polymer layer is enabled upon electroding the layer.

**Example 12: Detection of short strands of DNA using direct electrical measurements**

30 In Fig. 21(A) two conducting electrodes **502** are defined on an insulating substrate **501**. In Fig. 21(B) a monolayer of short, single-stranded

oligonucleotides **503** is constructed in the gap between a pair of electrodes **502** of the assay device. The sequence of the oligonucleotides is complementary to the sequence of the target to be deleted. The oligonucleotides have a dideoxy base at their 3' terminus and are therefore  
5 incapable of being extended with nucleic bases by use of transferase.

Fig. 21(C) shows that upon contacting said assay device with the sample, the target oligonucleotide **504** binds to the recognition moiety **503** thus forming a recognition group (double-stranded DNA) **505**. Different post-hybridization treatments such as washing at different temperatures and  
10 different salt concentrations ensure high selectivity in duplex formation.

In step (D), the assay device bearing the DNA duplex is contacted with a solution containing transferase and biotinylated bases which induces the elongation of the DNA skeleton at the 3'-deoxy site **506**.

In a subsequent step (step E), the assay device is exposed to a  
15 solution containing gold colloids coupled to streptavidin units **507**. The resulting assay device bears DNA molecules with pendant gold colloids **508**.

In the step step (step F), said assay device is exposed to a solution containing hydroquinone and  $\text{Au}(\text{SCN})_2$ . Gold is deposited only on metal surfaces that act as catalysis centers. The colloids grow and merge to  
20 form a conductive path **509** bridging the two gold electrodes. The detection of current at applied bias signals the presence of the target DNA sequence in solution.

In the absence of the target DNA sequence, no recognition group is formed between the electrode pair and no gold particles bind between  
25 the electrodes. The absence of metal nucleation centers prevents the formation of a conductive path between the electrode pair. The absence of electric current upon induced bias signals the absence of the target in the sample.

**Example 13: Detection of an Antibody by an Antigen or *vice versa***

The recognition moiety in this case is an antigen or an antibody selective to its antibody or antigen, respectively. The recognition moiety is attached to the electrode(s) by one of a variety of different methods, for example, by complexing it with another group that can bind to gold, by attaching a thiol group, etc; or it can be directly covalently linked to the electrode. In many cases van der Waals forces are sufficient to ensure binding of the recognition moiety to the electrode. The target may be attached to a modifier which eventually serves to bridge the gap between the electrodes.

10 For example, an antigen can be attached to an end of a DNA fiber and bind to its antibody on the electrode with this modifier attached. The other side of the DNA fiber can selectively bind to another electrode in the assay set, or it can be non-selectively attached to it. Substrate passivation, electrode definition, metalization and detection follows the general principles outlined above, e.g.

15 in Example 8.

**Example 14: PCR and other methods allowing the introduction of modified nucleotides**

20 The introduction of modified bases into DNA or RNA fibers may help in constructing the detection system. Examples of modified nucleotides are: biotin derivatized nucleotides or nucleotides with prime amine groups connected to them. There are different standard molecular biological techniques allowing the introduction of modified nucleotides in

25 specific location along an existing DNA or RNA fiber or constructing copies of a nucleic acid template with a complete sequence of modified nucleotides. For example, the polymerase chain reaction (PCR) technique can be used to amplify a nucleic acid template with modified nucleotides. In this case the modified bases serve as the nucleotides in the PCR solution (mixed with

30 unmodified bases or not) and together with the provided primers (the latter can be synthesized with the same modified nucleotides if necessary)

facilitating the amplification process. Alternatively, the method of random priming allows the replacement of nucleotides with modified nucleotides. In some cases ligation of the polymerization products along the template is required to ensure a continuous fiber. Another possibility is to use one of the  
5 stranded polymerases (e.g. a Klenow fragment) to fill gaps along double-stranded DNA fibers or to fill sticky-ends with modified bases. Alternatively, DNA terminal transferase can be used to attach modified bases to the 3' side of a nucleic acid fiber (single or double-stranded). In cases  
10 fiber they can be used to attach the fiber specifically to other groups, e.g. a thiol group, a streptavidin or another nucleic acid fiber, etc.

Constructing a complete sequence of a nucleic acid fiber with modified nucleotides allows to achieve highly selective metalization of the bridges. By this method, the modified bases attach specifically groups or  
15 complexes that can serve as nucleation centers to catalyze metalization prior to detection. For example, nucleotides derivatized with amine groups can bind specifically tiny gold clusters or colloids that serve as well defined nucleation centers for gold or silver (and many other metals) deposition for yielding a conductive bridge. Another examples is to use bases derivatized with biotin  
20 and to attach colloids or gold clusters coated with streptavidin along the DNA fiber. These colloids or gold clusters again can serve as nucleation centers for the metalization process.

#### **Example 15: *In situ* PCR on electrodes or between electrodes**

25 *In situ* polymerase chain reaction (PCR) is a relatively recent technique used usually to detect minute quantities of DNA or RNA in tissue sections or intact cells. It uses the high selectivity of hybridization techniques, allowing, for example, to correlate a specific sequence with a tissue section, with the amplification power of the PCR allowing the many fold increase in

detection sensitivity (for a recent review of the technique see: *In Situ* Polymerase Chain Reaction and Related Technology, edited by J.Gu. Eaton Publishing, 1995). This technique can be employed for specific target amplification, on the substrate or electrodes of the assay device, for example,  
5 a DNA sequence present in a minute concentration.

In a first step, the target in front of the sample binds to the specific recognition moiety immobilized on a substrate member situated between an assay set of electrodes. e.g. by hybridization with a complementary DNA sequence fixed to the substrate by e.g.  
10 biotin-streptavidin, amine-thiol, etc. A solution containing appropriate primers, optimized concentration of bases and the appropriate buffers and one of the standard PCR polymerases (e.g. a Taq polymerase) is added and then a thermal cycle can be started. In that case the assay device is placed in a temperature control apparatus allowing to control and modify its temperature  
15 in fast and automatic way. The template is now specifically duplicated in each cycle to form new templates for the next cycle, hence exponential amplification is possible. The new templates are generated *in situ*, and may attach to the substrate to form a network with the original DNA target. This may be achieved by non specific binding of the long DNA fibers or by  
20 specific binding. For example, by photoactivating, at the end of each cycle, a moiety group attached to the newly formed templates leads to their binding to other complexes on the substrate or to other templates already attached to the substrate. This binding should not interfere with the possibility to bind the primers at the next cycle for further amplification. After sufficient cycles (e.g.  
25 30) a network of DNA fibers, exact copies of the original target, attached to the substrate, fills the gap between an assay set of electrodes. Metalization process then follows, according to one of the techniques specified above, allowing eventually an electric detection of the formation of this network bridge.

One modification to the above is the possibility to carry the amplification procedures between two substrates (e.g. closely spaced glass slides or nylon membranes) that will force newly formed templates to stay *in situ*. For example, a filter membrane with the proper cutoff, allowing the  
5 passage of primers, bases, polymerase and buffers but not long templates can serve for that purpose. In this case all the necessary ingredients can be continuously fed through the membrane ensuring no escape of the templates. Such a semi-permeable membrane also enables efficient washing before metalization and detection.

10

**Example 16: Ligase chain reaction (LCR) on electrodes or between electrodes**

The ligase chain reaction is a technique for amplifying a  
15 specific sequence by ligating at each cycle two subsections of a template. By using a thermocycler identical to the one used for the PCR technique, denaturization of the templates followed by annealing of subsections that exactly match the template to form a continuous nucleic acid fiber with a single or multiple nicks. These nicks are ligated by a special ligase that work  
20 at high temperatures (e.g. pfu ligase). This technique can be applied *in situ*, similar to Example 15 above. An example for a possible amplification is to ligate two short subsections, to a strand not long enough to bridge the gap between electrodes. Because of the amplification power of this technique (again in each cycle newly formed ligated fibers serve as templates for the  
25 next cycle), it allows to form a bridge across electrodes made specifically from copies of the target to be detected which is introduced originally in only a minute concentration.

**Example 17: Enzyme-substrate or protein-molecule as possible affinity groups**

To allow electrical detection of small molecules such as an enzyme (or its substrate) or a protein in some cases they may be attached to a modifier. For example, biotin can be attached to a nucleic acid fiber to enable the detection of avidin or streptavidin. In some cases the modifier can be a synthetic polymer. Another example is the use of a conducting polymer as a modifier facilitating electrical detection in later stages.

10

**Example 18: Bacterium detection**

The target to be detected in this case is a bacterium. The recognition group can be, for example, an antibody to a specific antigen on the bacterium. Alternatively, biotin-avidin or other specific binding between a molecule or a supramolecular structure on the bacterium membrane and a proper recognition moiety on the electrodes or on the substrate between electrodes is possible. The bacterium will form a bridge across electrodes. Metalization then facilitates electrical detection. An electric field may assist in directing the bacterium to the proper location on the substrate or electrodes. In some cases, it is possible to use ionic currents (alone or in combination with electronic currents) through the bacterium (e.g. using the bacterium natural ionic channels) for electrical detection. Electric or magnetic fields or light can be used as tweezers for bacteria, trapping them between electrodes prior to their metalization and electrical detection.

25

**Example 19: An array for multiplexed assay of a target**

Fig. 22A depicts a device designed for a multiplexed assay of a target or targets in a sample. The device comprises vertical conducting lines 601, 602, 603, 604 and horizontal conducting lines 611, 612, 613, 614. The lines are insulated from each other at the intersection points. In the

30

vicinity of each intersection point, a diode, (one is represented as 620) connected, at its anode side to the horizontal line. The cathode is connected to an electrode, (one is represented as 631). Another electrode, 632, opposite to the first one, is connected to the corresponding vertical line.

5           Each electrode is attached to a recognition moiety. The various electrode pairs may be designed to detect the same target for example for a quantitative assay of the concentration of the target in the sample. Alternatively, the various electrode pairs may be found to detect different targets, for example for sequencing by hybridization (SBH) purposes. Where  
10 the device is interacted with a sample containing the targets, a bridge is formed between some electrode pairs. Upon functionalization, the bridge is made conductive as described previously, for example 641, 642, 643 in Fig. 22(B), thus connecting the corresponding diode cathode to the corresponding vertical line.

15           In the example depicted in Fig. 22(B), functionalized bridges were formed at 641, 642, 643, thus connecting diode 621 to the vertical line 601, diode 622 to the line 601, and diode 623 to line 603. The diodes are introduced in order to eliminate "crosstalking" between the various horizontal and vertical lines.

20           In order to detect the functionalized bridges, the bias on the various lines is scanned in the following way and the current is measured. In the first step, a positive potential  $V$  is applied to the horizontal line 611 and to all vertical lines but 601. Simultaneously, a zero potential is applied to the vertical line 601 and to all horizontal lines except 611. It is easy to see that  
25 under these conditions current will flow only when a functionalized bridge is formed between electrodes 631, 632. In the next step, the potential on line 611 is set to zero while the potential on line 612 is set to  $V$  (all other potentials being the same).



In the case of Fig. 22(B), the functionalized bridge **641** will lead to current indicating its existence. The existence of other functionalized bridges will not affect that result.

Scanning all horizontal and vertical lines this way pinpoints all  
5 functionalized bridges. Diodes are essential in this scheme. Replacing them with a simple conductor will result e.g. for the case depicted in Fig. 22(B), in a current flow through both **641** and **642**. The diode **622** prevents such a misleading current path.

The electronic module to be used in such an array may contain  
10 the necessary multiplexing capabilities.

CLAIMS:

1. An assay device for detecting a target in a sample, comprising one or more assay sets each comprising at least two electrodes and a  
5 recognition moiety capable of specific binding to the target, the arrangement being such that binding of the target to the recognition moiety permitting the formation of a bridge between at least two electrodes of an assay set for establishing electric conductance between the at least two electrodes.
2. An assay device according to Claim 1, wherein each assay set  
10 consists of two electrodes.
3. An assay device according to Claim 1 or 2, wherein a recognition moiety is immobilized on at least one electrode of each assay set.
4. An assay device according to Claim 3, wherein at least two  
15 electrodes of the assay set have a recognition moieties immobilized thereon. these recognition moieties being the same or different, bind specifically to the same target.
5. An assay device according to Claim 3 or 4, wherein the recognition moiety is immobilized onto the electrode by means of a linker conjugated or complexed with the recognition moiety and attached by a  
20 covalent or non covalent bond, to the electrode.
6. An assay device according to Claim 1 or 2, wherein the recognition moiety is immobilized on a carrier substrate which is other than the electrode.
7. An assay device according to Claim 6, wherein the bridge is  
25 formed by treatment following binding of the target to the recognition moiety.
8. An assay device according to any one of the preceding claims, wherein electric contact between the electrodes of an assay set is established by electric functionalization of the bridge.

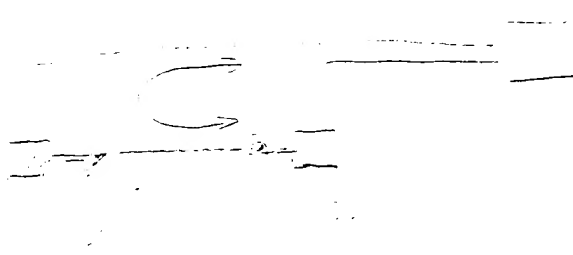
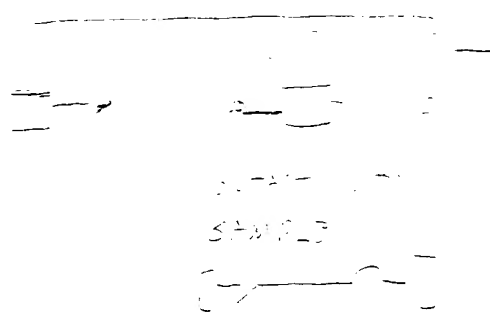
9. An assay device according to any one of the preceding claims, comprising a plurality of assay sets of electrodes.
10. An assay device according to Claim 9, wherein all assay sets of electrodes are for assaying the same target.
- 5 11. An assay device according to Claim 8, wherein different assay sets of electrodes or different groups of assay sets are for assaying different targets.
12. An assay device according to Claim 11, for simultaneous determination at different targets in a sample.
- 10 13. An assay device according to any one of the preceding claims, wherein the target is a nucleic acid sequence and the recognition moiety is an oligonucleotide comprising a sequence which is complementary to at least portion of the target.
14. A system for assaying of a target in a sample, comprising at  
15 least one device according to any one of the preceding claims, and an electric or electronic module for determining electric conductance between at least two electrodes of an assay set.
15. A system according to Claim 14, wherein the electric or  
20 an assay set. electronic module measures the current flow between different electrodes of
16. A system according to Claim 14, wherein the electric module is used to direct the recognition moiety and/or the target to their position in relation to the assay set by use of electric field.
17. A system according to any one of Claims 14-16, wherein the  
25 electric or electronic module measures potential different between the electrodes of a set.
18. A system according to any one of Claims 14-16, wherein the electric or electronic module measures current passing between electrode of a set.

19. A method for assaying a targeted DNA sample comprising:
- (a) providing an assay device according to any one of Claims 1 to 13.;
  - (b) contacting said assay device with said sample to form, at least  
5 one bridge between at least two electrodes of an assay set;
  - (c) providing conditions or treating the device such so as to allow current to flow across said bridge; and
  - (d) deforming electric conductance between different electrodes of an assay set, conductance above control level indicating presence of said  
10 target in a sample.
20. A method according to Claim 19, wherein conductance in step (d) is determined by delivering current and measuring potential drop.
21. A method according to Claim 19, wherein conductance in step (d) is determined by applying potential and measuring current.
- 15 22. A method according to any one of Claims 19-21, wherein step (c) comprises functionalizing the bridge by depositing or forming thereon an electrically conducting substance.
23. A method according to any one of Claims 19-22, wherein the bridge forms as a result of binding of the target to the recognition moiety.
- 20 24. A method according to any one of Claims 19-22, wherein the bridge forms by treating the assay device following contact with the sample.
25. A method according to any one of Claims 19-24, wherein said assay device comprises a plurality of assay sets of electrodes and said electric measurements are performed separately for each assay set.
- 25 26. A method according to any one of Claims 19-25, wherein the electric measurement involves determination of potential/current relationship which is indicative to the number of formed bridges, which is in turn indicative of the quantity or concentration of the target in the sample.

27. A method according to Claim 25, wherein all assay sets of electrodes are for assaying the same target.
28. A method according to Claim 27, wherein the number of assay sets of electrodes were electrical functional bridges form. is an indication of  
5 the concentration of the target in the sample.
29. A method according to Claim 28, wherein the number of sets of electrodes where functional bridges formed is measured as a function of time.
30. A method according to Claim 22 or 23, wherein different assay sets of electrodes or different groups of assay sets are for assaying different  
10 targets.
31. A combination of reagents for use in a method according to any one of Claims 19-30.
32. A combination of reagents according to Claim 31, for use in functionalizing said bridge to render it electrically conductive.
- 15 33. A kit for use in a method according to any one of Claims 19-30, comprising an assay device according to any one of Claims 1-13 and a combination of reagents according to any one of Claims 31 or 32.
34. A kit for use in a method according to any one of Claims 19-30, comprising a system according to any one of Claims 14-18 and a combination  
20 of reagents according to any one of Claims 31 or 32.

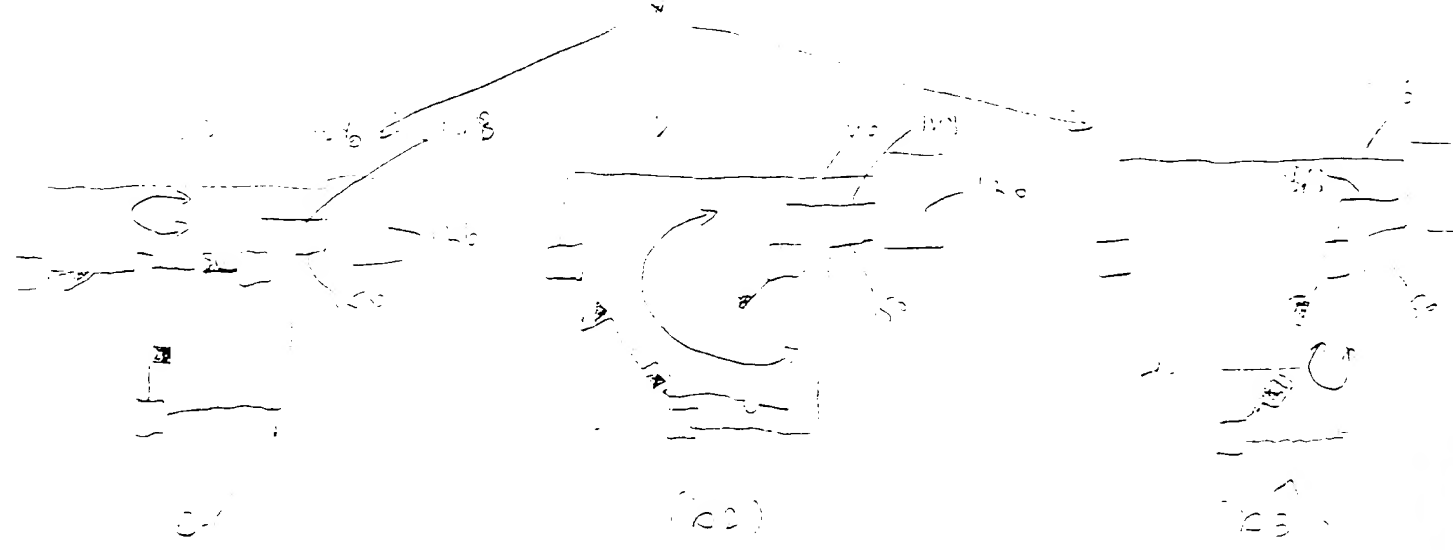
For the Applicants,  
**REINHOLD COHN AND PARTNERS**  
By:

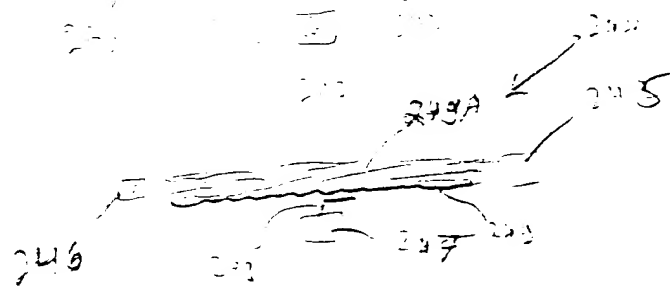
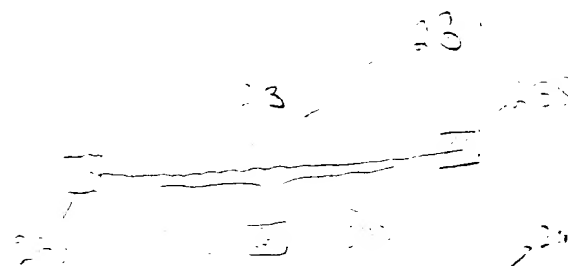
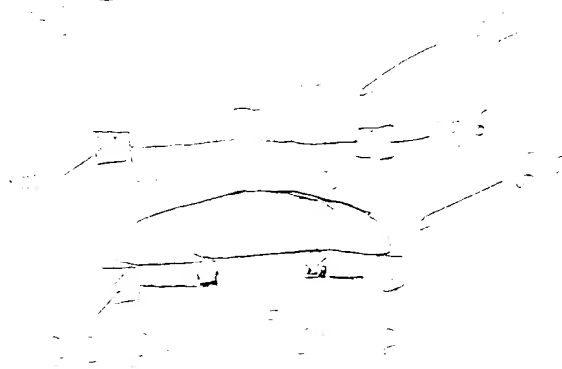




CONTACT WITH  
SAMPLE

Fig. 2





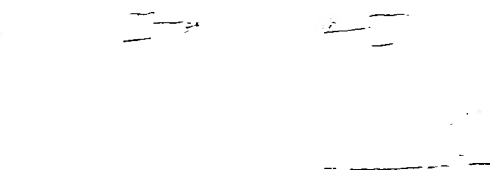


Fig. 5

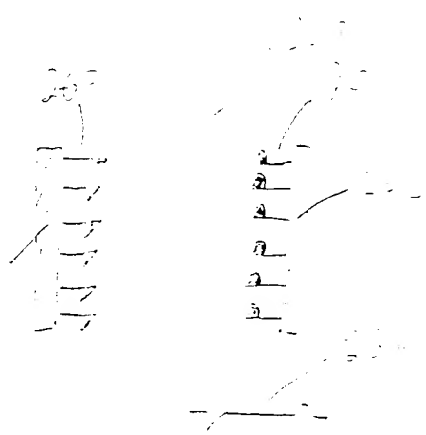
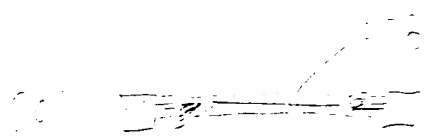
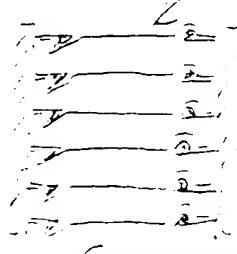
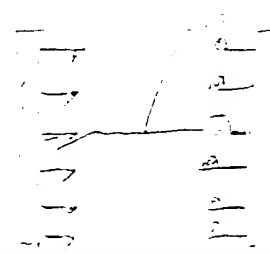
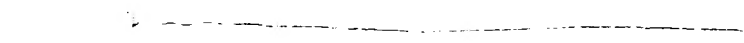


Fig. 5







2013

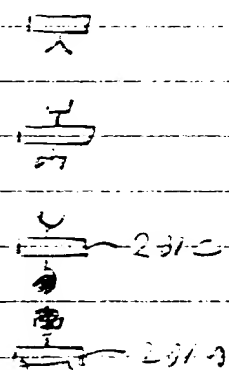
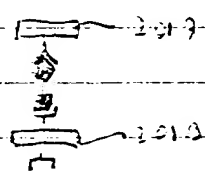
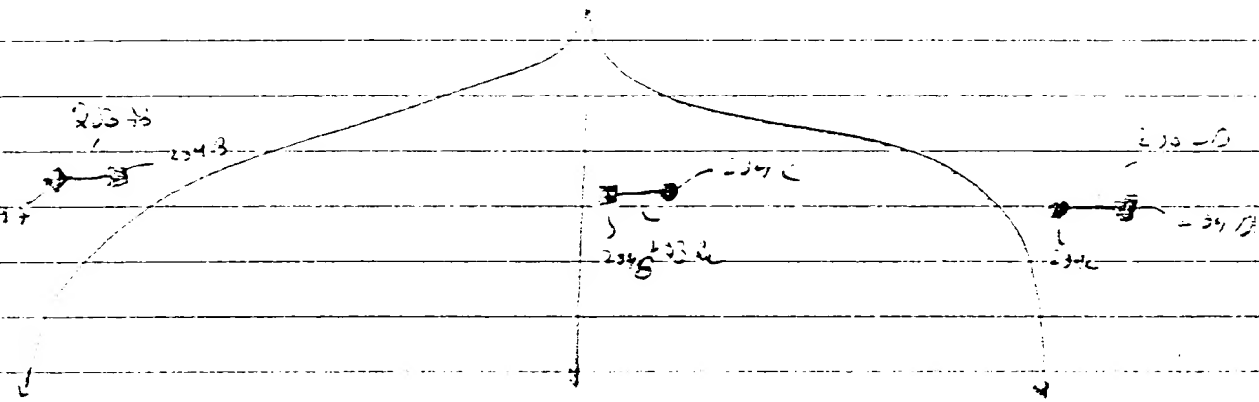
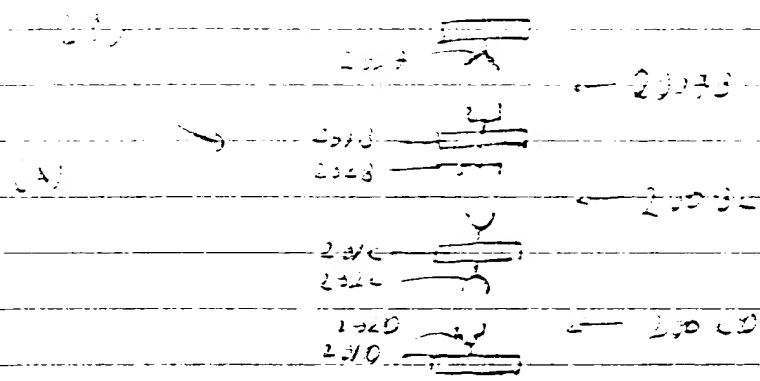


Fig. 9

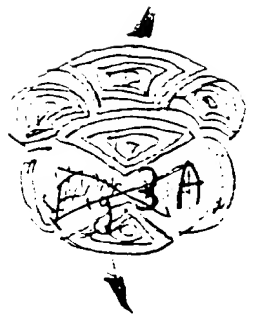
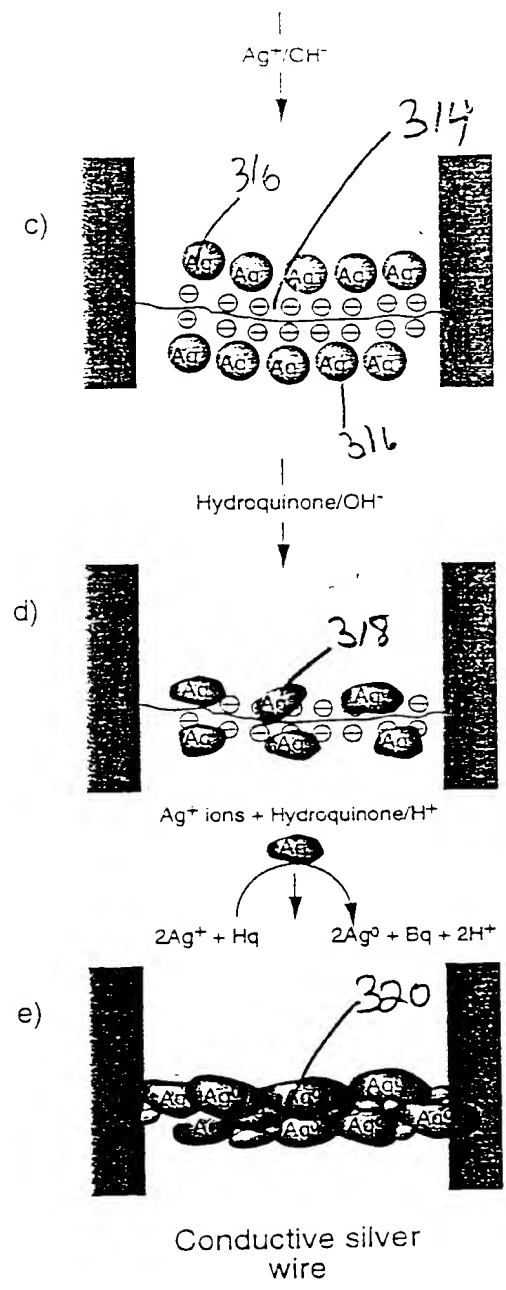
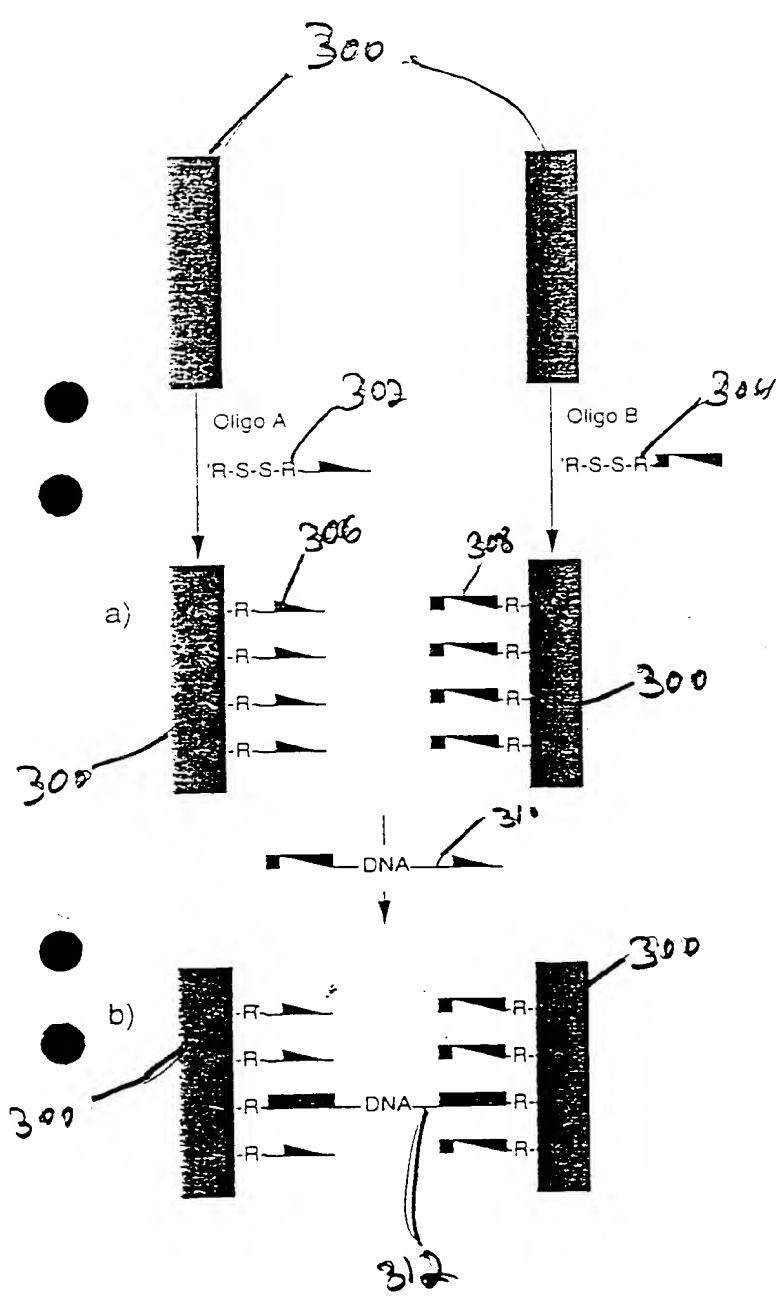
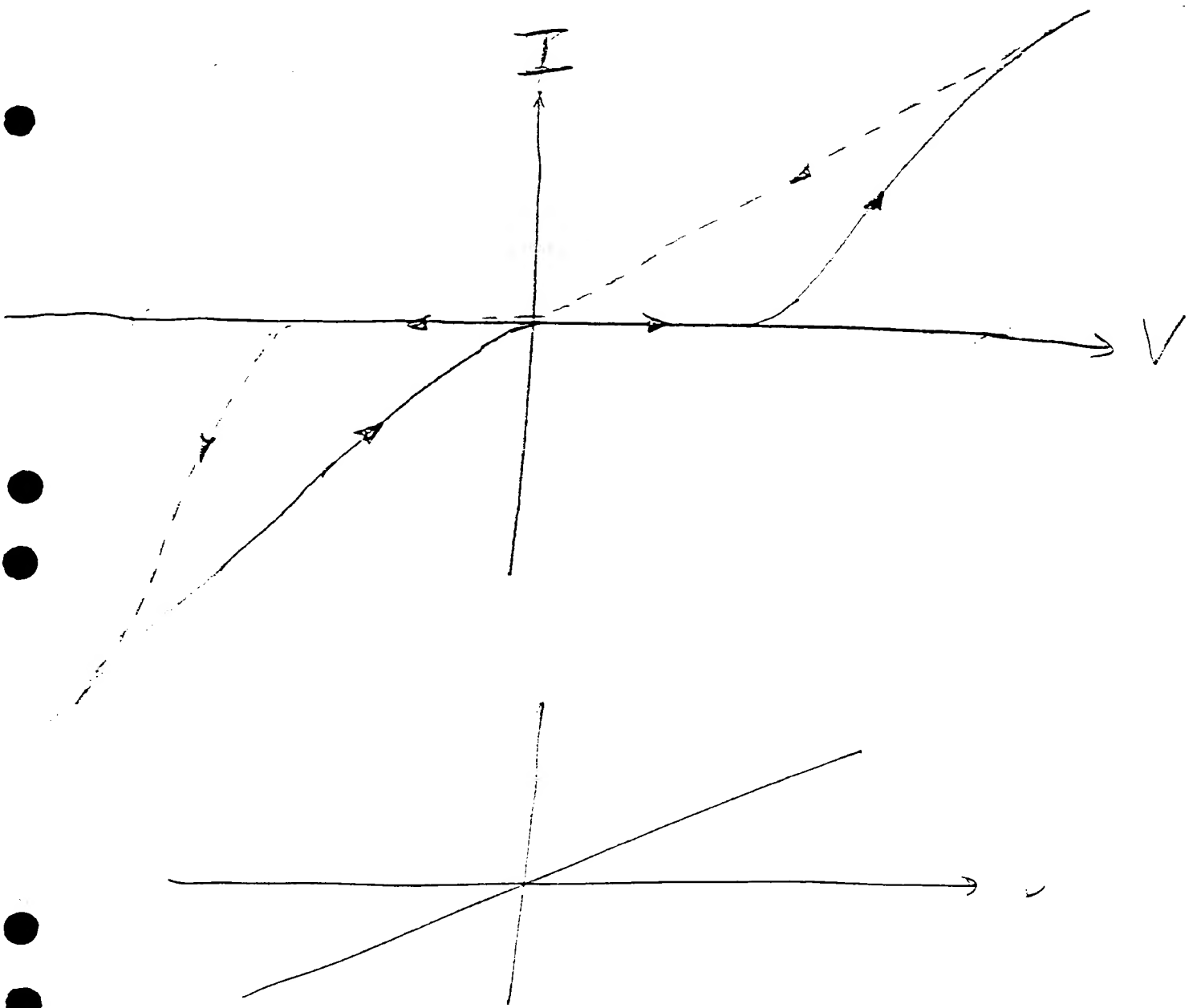
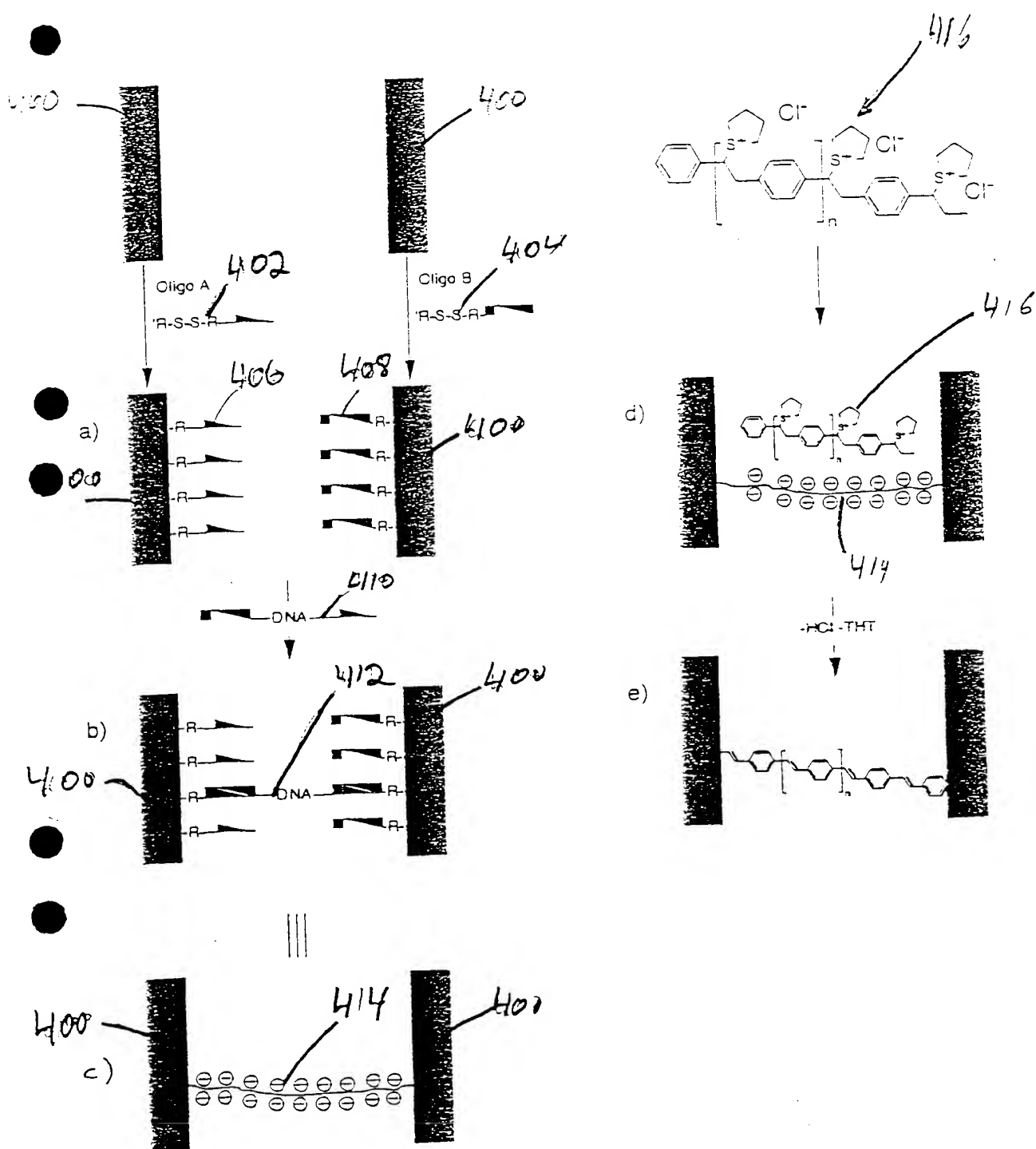


Fig. 10  
Fig. 1A



~~Fig 3B 1B~~ 10

Fig. 11



(7)

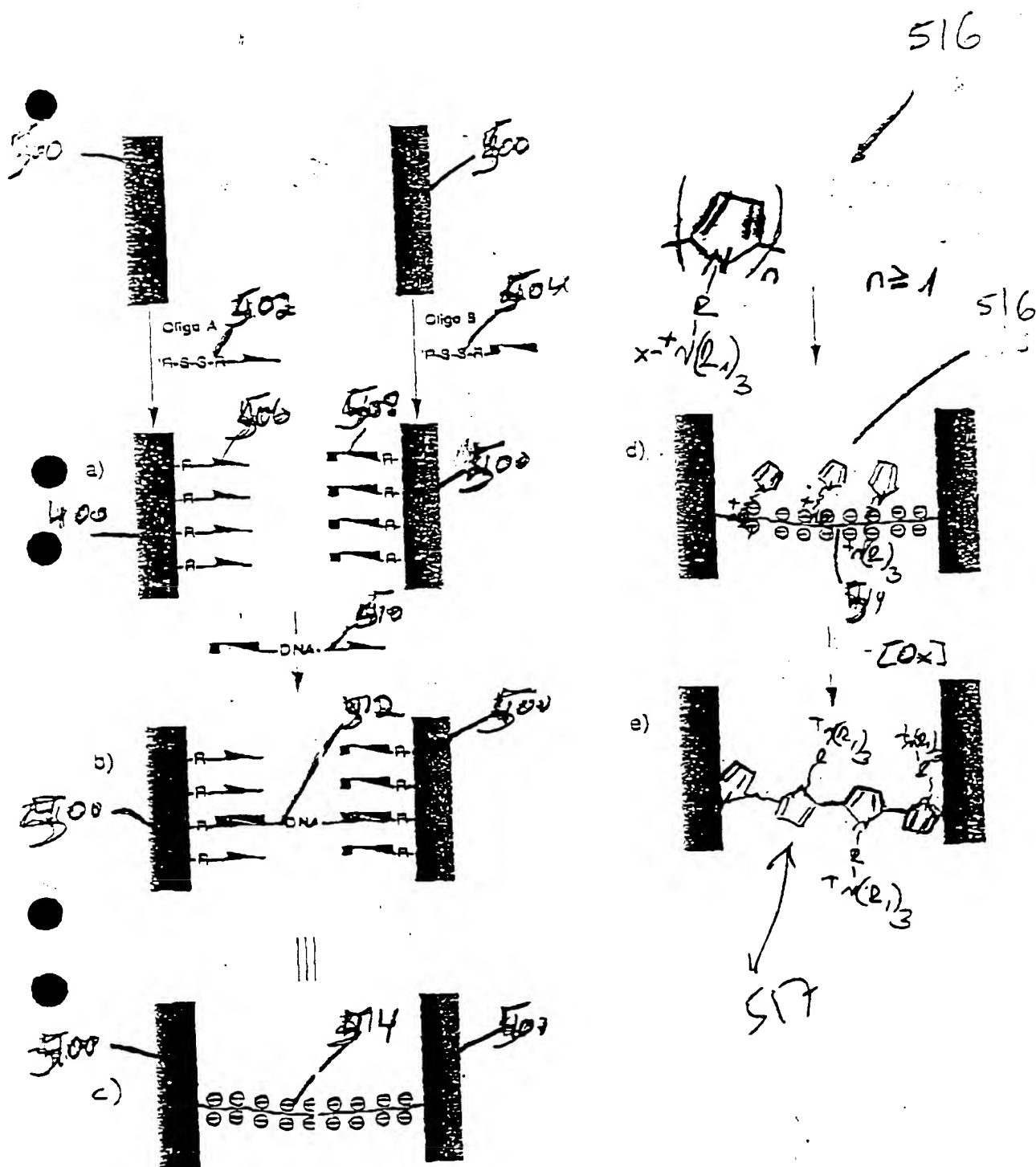


Fig. 12

Fig. 13

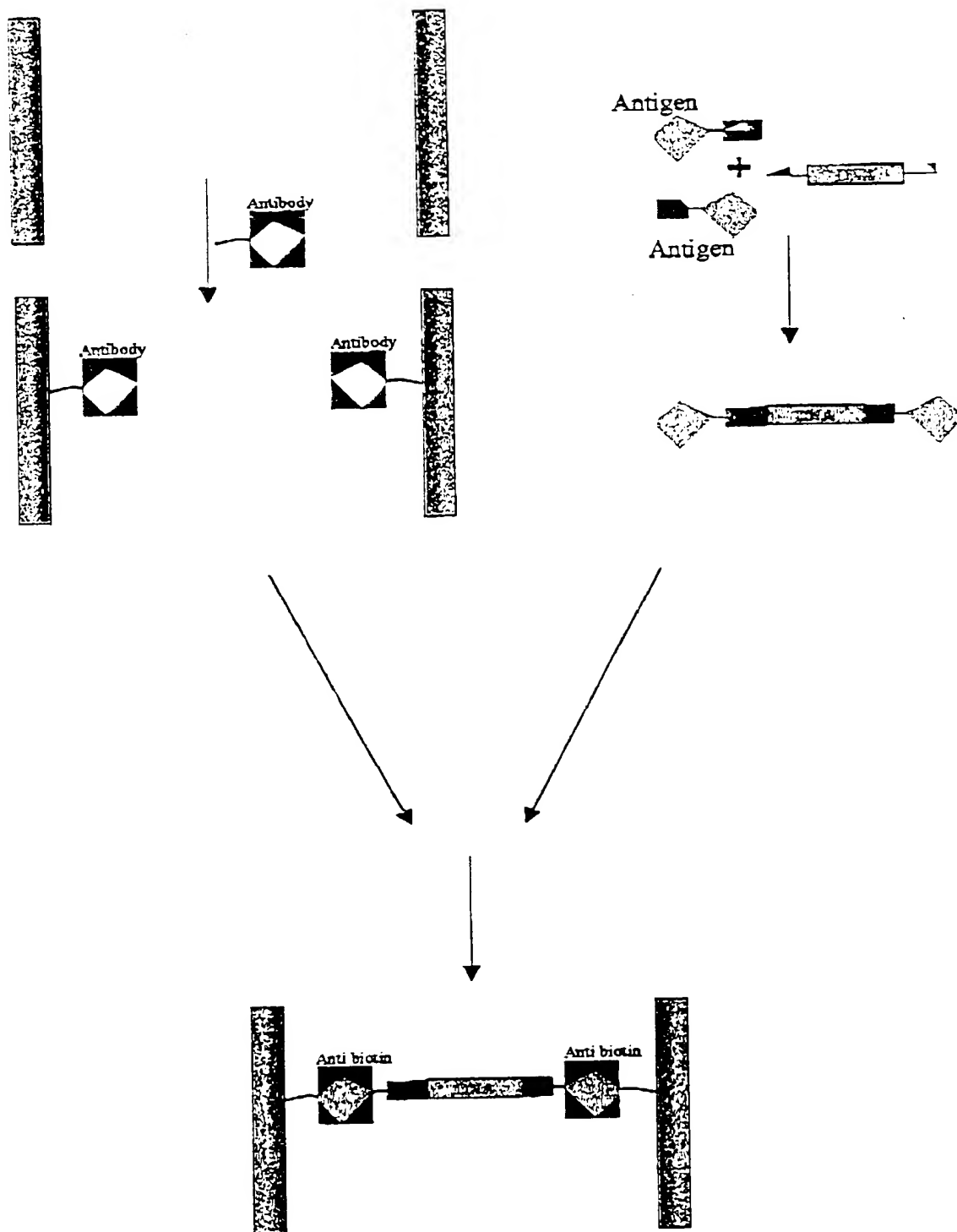
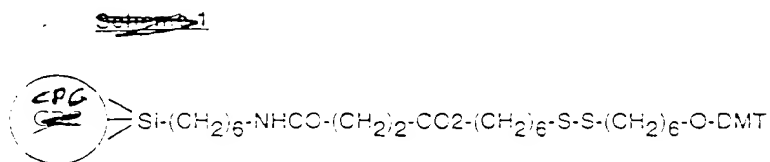
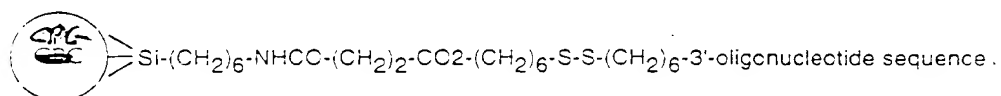


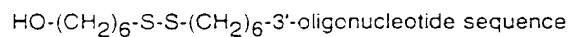
Fig. 8134



Building the sequence

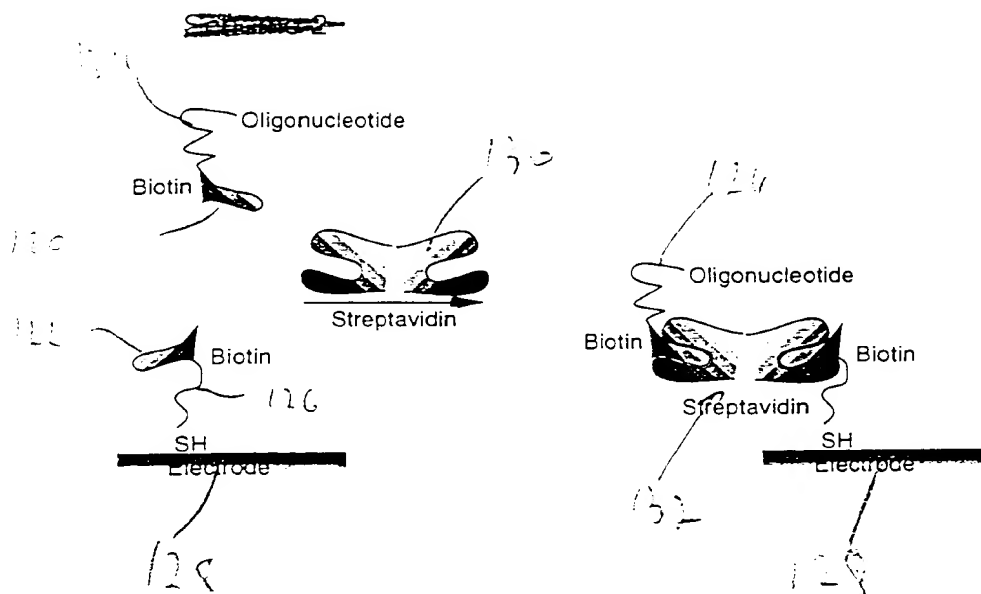


Base



~~Fig. 15~~

Fig. 16



~~Fig. 15~~

Fig. 15





~~Fig. 16~~ ~~Fig. 15~~

Fig. 17

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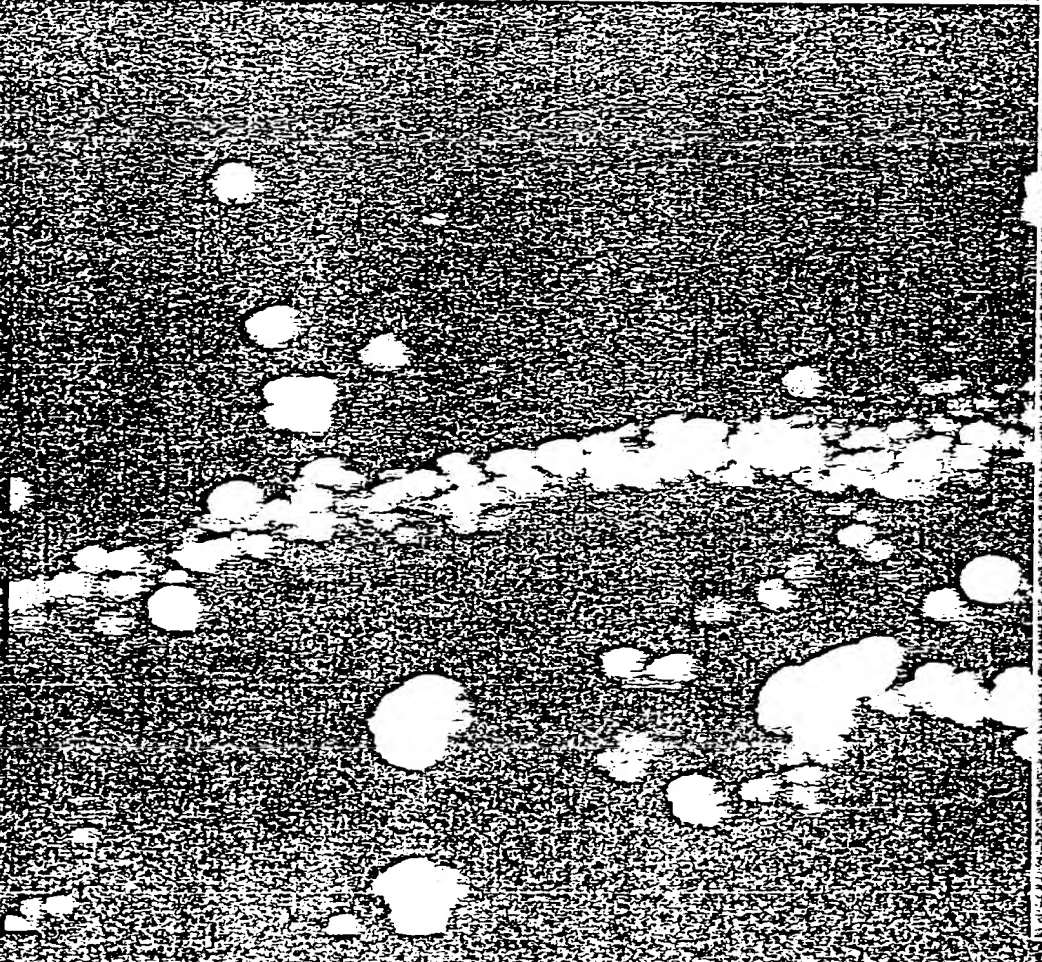
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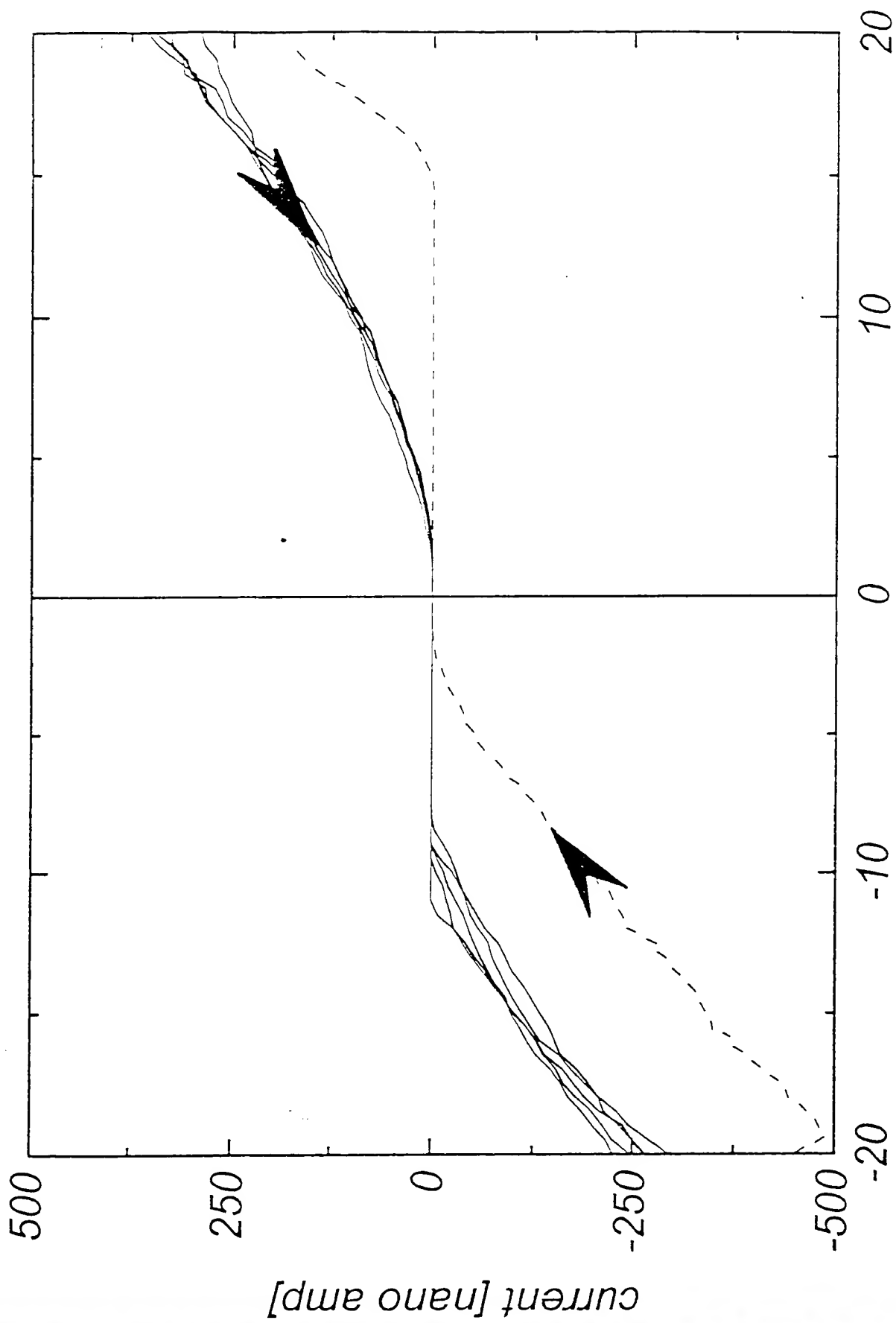
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~~Fig. 12A~~

Bias [volt]

~~Fig. 12A~~

Fig. 4a

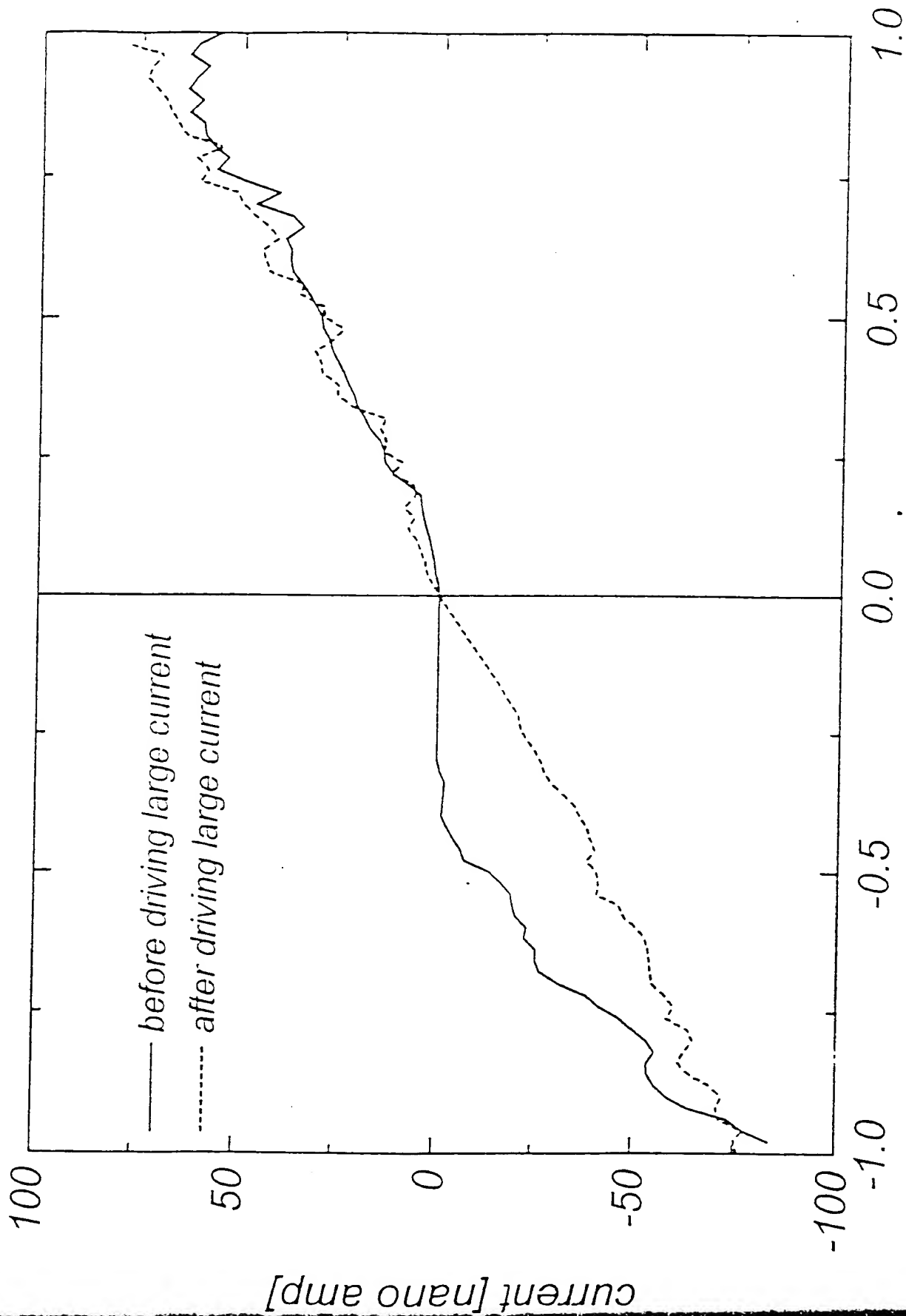


Fig. 4B

Fig. 4B

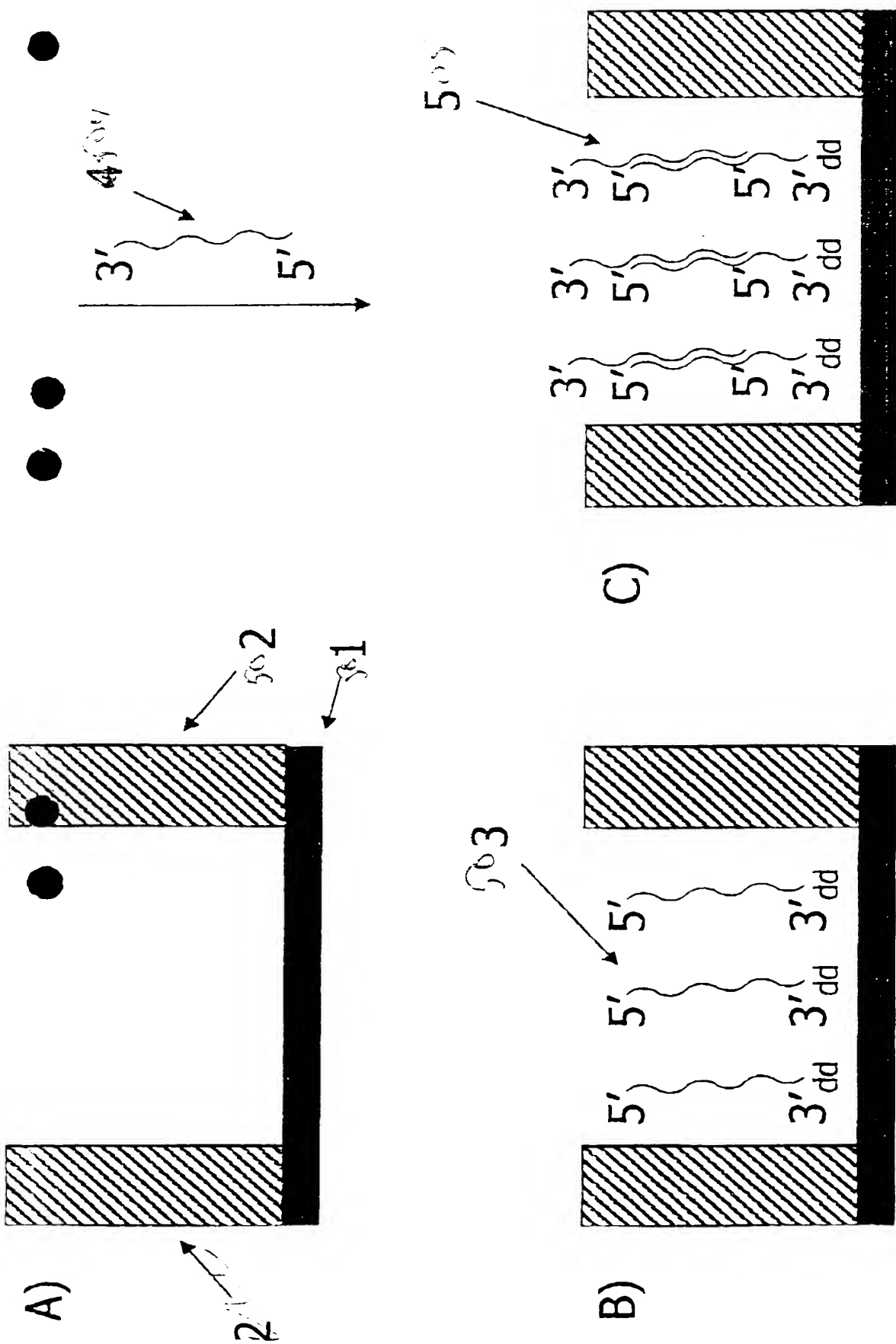
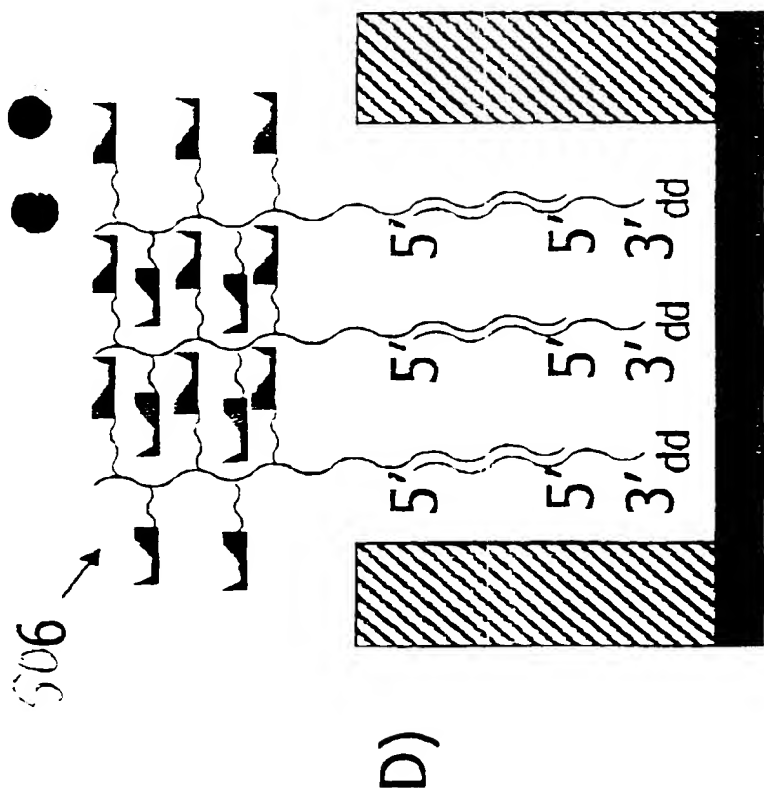


fig. 20: 110



E)

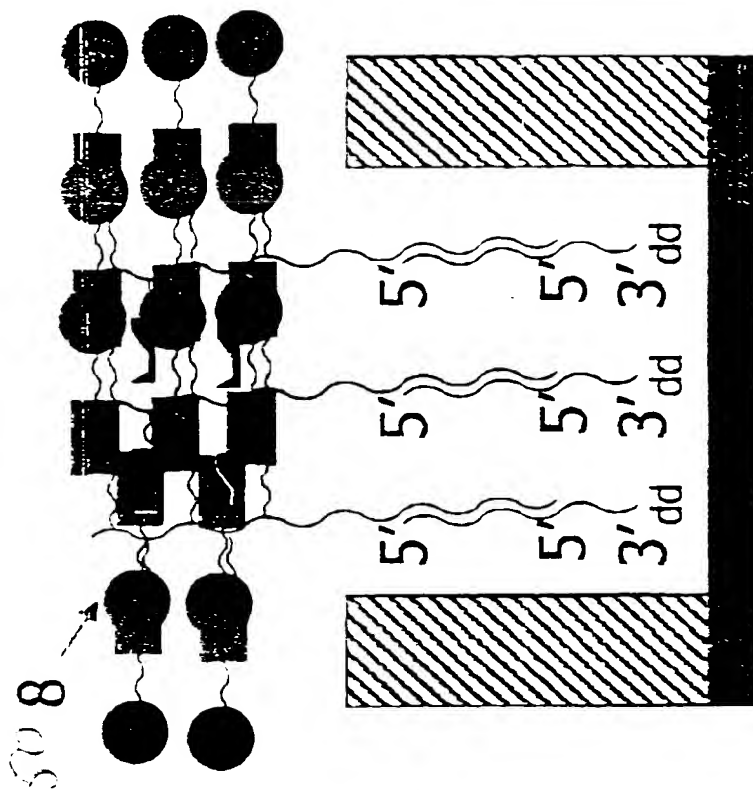
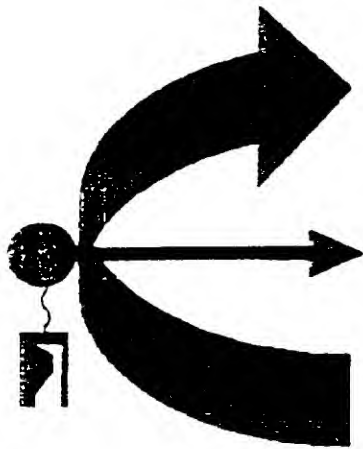


fig 21 (contd.)

fig. 20/21



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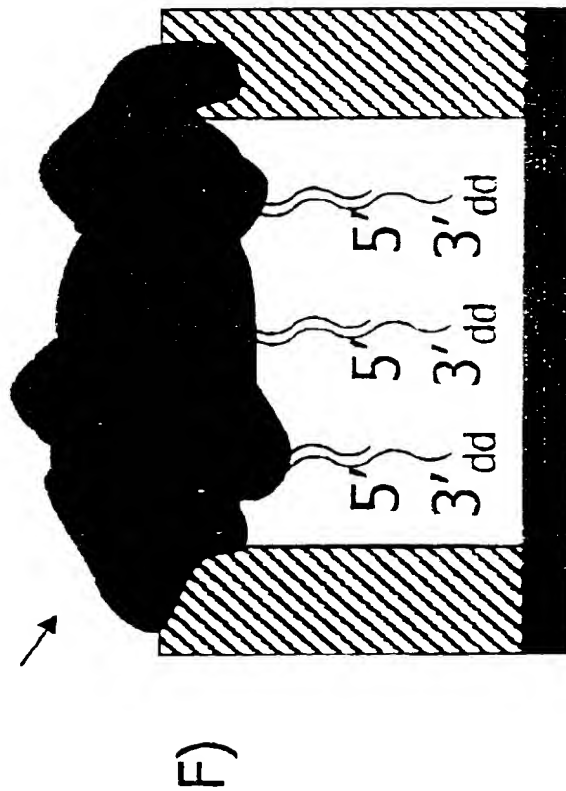
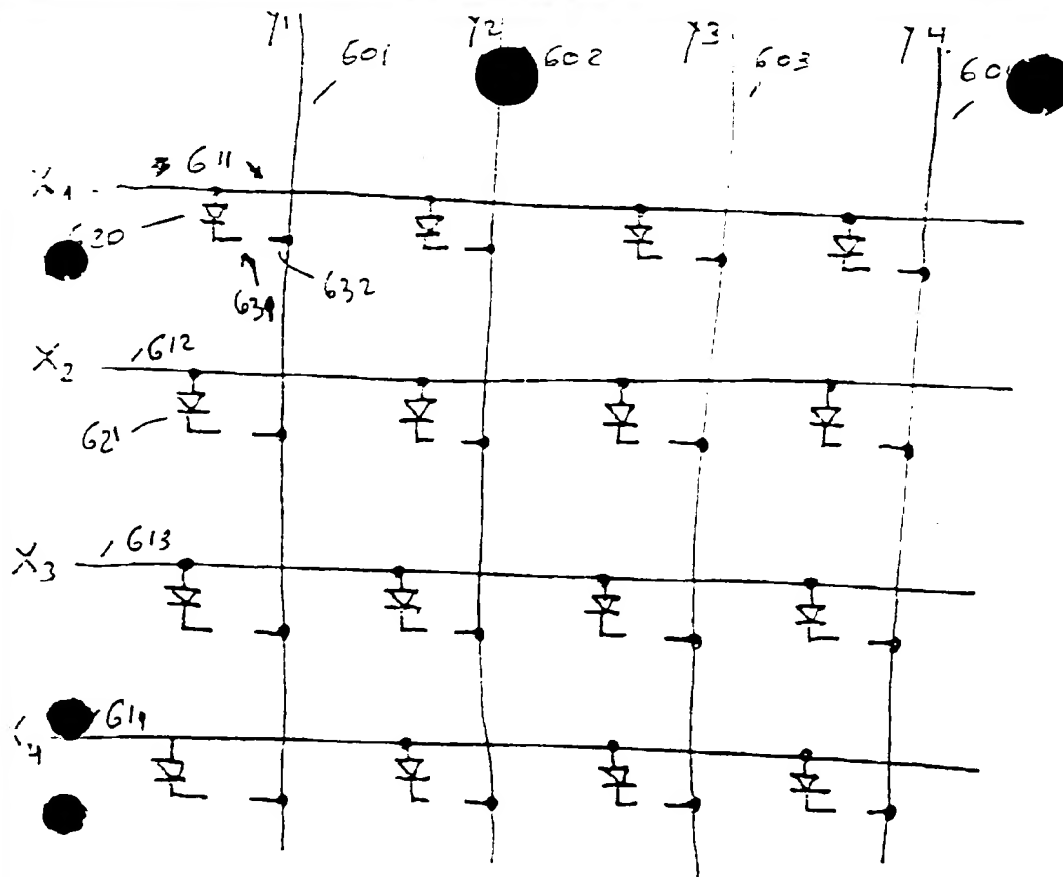
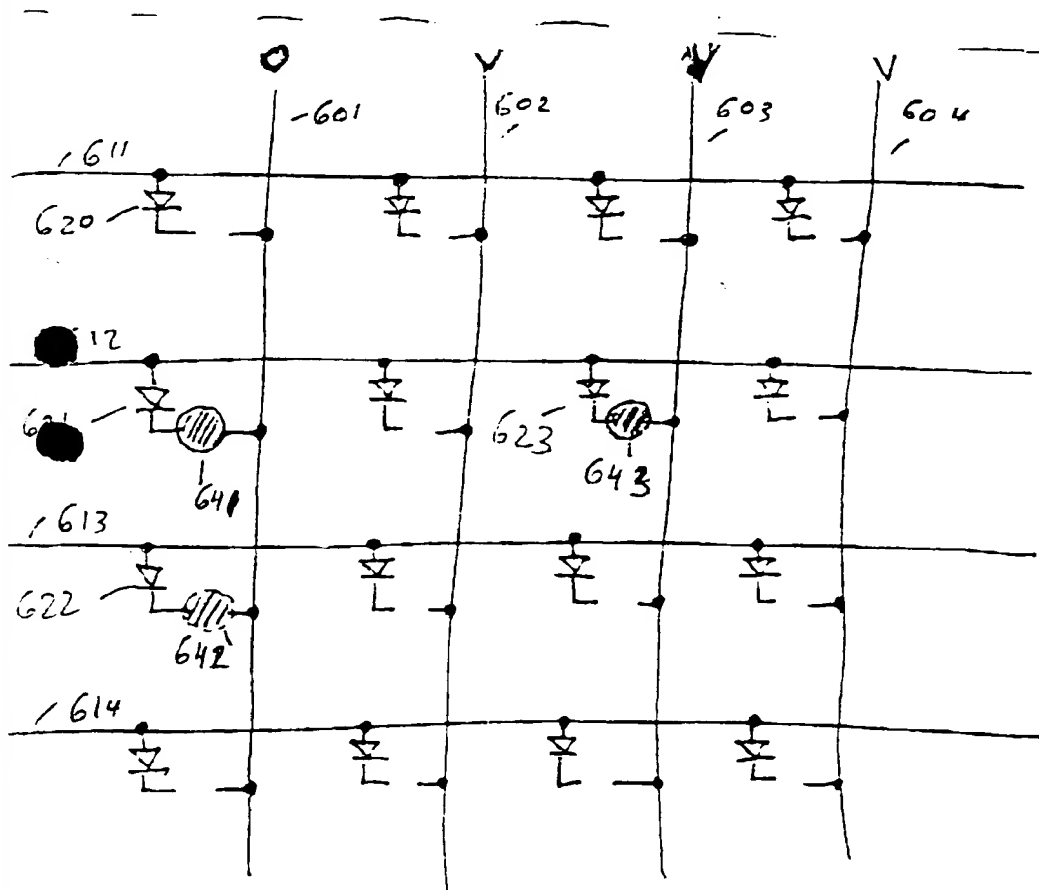


Fig. 20-21

(Fig. 21 - cont.)



(a)



(b)

Fig. 2d